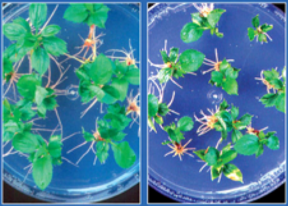
Plant Propagation by Tissue Culture 3rd Edition

Volume 1. The Background

Edited by
Edwin F. George, Michael A. Hall and Geert-Jan De Klerk



Springer

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3rd Edition

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Volume 1. The Background

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Preface

It is now more than twenty years since the first edition of this work appeared and nearly fifteen since the second. Whilst much of the information in those editions has stood the test of time, inevitably, because of the pace of research, a new edition is clearly timely.

This is true, not only because many more species have been the subject of propagation studies, but because the background to the field – with which this volume deals – has changed almost out of all recognition. In particular, our knowledge of plant development, genetics physiology, biochemistry and molecular biology has expanded exponentially – often through work on mutants of *Arabidopsis* – and opened up many new avenues for the plant propagator to explore. Equally, the commercial significance of plant propagation has increased significantly. As an example, in the second edition there was a single chapter on plant growth regulators – in this there are three, reflecting the fact that not only is there more information on those PGRs we recognised in 1993, but that several new ones are now known. Equally, fifteen years ago we knew little of the molecular basis of plant development e.g. flower and shoot development, in this edition it has merited a whole chapter, much of which relates to discoveries in the last decade.

Because of these factors, it was felt that a different approach was required for this edition. The second edition was researched and written by Edwin George alone but it would now be very difficult for a single author to gain the breadth of expertise necessary to cover all the relevant aspects of this many-faceted subject. Hence, it was decided to adopt a multi-author approach, with chapters written by experts in their fields. These build upon the sound framework of the previous editions (which those with a knowledge of the previous works will recognise). Many sections of the previous work have been retained, but inevitably, apart from up-to-date reference lists, the text has undergone major revision in many areas.

Like the previous edition, the current one will appear in two volumes, but coverage has been extended and the order in which subjects are covered has been changed. Therefore, some topics, previously covered in Part 1, will now be discussed in Part 2. The ethos of the work is, as before, to produce an encyclopaedic text.

The first initiative to begin the new revision of *Plant Propagation by Tissue Culture* was made by Prof. A.C. Cassells and the editors are grateful to him for his early leadership. No work of this size can be accomplished successfully without much goodwill and hard work by the contributors, and to them the editors express their deepest thanks. We also express our sincere thanks to all those who have allowed us to use their material in diagrams and illustrations. We are very appreciative of the hard work by Dr. Susan Rafferty-McArdle of University College Cork in formatting the text, and to Dr. Jacco Flipsen of Springer for his support.

Edwin George
Mike Hall
Geert-Jan de Klerk
May 2007

Biographical Notes on Contributors

Chapter 1.

Edwin. F. George trained as a botanist at Imperial College, London and subsequently gained a PhD, working on breeding and selection of sugar cane at the Mauritius Sugar Industry Research Institute. He was later employed by ICI Ltd. and Plant Protection Ltd. to study plant growth regulating compounds and subjects for corporate research. He finally became an independent consultant and researched extensively into plant genetic engineering and especially plant tissue culture. This resulted in the books *Plant Culture Media, Vols. 1 and 2* (1987), and *Plant Propagation by Tissue Culture*. The latter work was first published in 1984 and then extensively revised and extended to two volumes in 1993 and 1996. The present book is based on the first volume of the 2nd edition of *Plant Propagation by Tissue Culture.* Dr. George prepared the diagrams for the current revision although he is now retired.

Chapter 2.

Pierre C Debergh is Emeritus-Professor of the University of Gent (Belgium) since 2004 and specialised in micropropagation since 1968. His major interest is in tissue culture (*sensu largo*) and horticulture applied to western and developing countries (Asia, Africa and the Carribean). He is editor of *Plant Cell Reports*; *Plant Cell, Tissue and Organ Culture* and the *South African Journal of Botany*. He is author of approx. 100 publications and supervisor of 35 PhD dissertations and more than 250 MSc dissertations.

Chapter 3.

Geert-Jan de Klerk is senior scientist in plant tissue culture since 1986, first in *The Centre for Plant Tissue Culture Research* in Lisse (Netherlands) and now in *Plant Research International, Wageningen University* (Netherlands). His main research interests concern plant developmental biology. He is editor-in-chief of *Plant Cell Tissue and Organ Culture* and editor of *Propagation of Ornamental Plants.*

Chapter 4.

Trevor A Thorpe was a PhD student of Toshio Murashige at the University of California, Riverside (USA). He was a Faculty Professor and now Professor Emeritus in the Department of Biological Sciences at the University of Calgary, Alberta, Canada. He retired in 1997 but is still an active researcher. His areas of interest include developmental plant physiology, experimental plant morphogenesis and micropropagation, mainly of woody plants. He was a former Chairman of the International Association for Plant Tissue Culture and former editor-in-chief of *In Vitro Cellular and Developmental Biology – Plant*.

Edward C Yeung was a PhD student of I Sussex at Yale University. He is an Assistant Professor in the Department of Plant Science at the University of Manitoba (Canada). His research interests are structural, physiological and biochemical ontogeny of plant embryogenesis and floral biology of orchids

Claudio Stassolla was a PhD student of Edward Yeung at the University of Manitoba, (Canada). His research is on plant somatic embryogenesis *in vitro*.

Andy V Roberts is Emeritus Professor in the School of Health and Biosciences at the University of East London (UK). His research interests are the use of *in vitro* methods for the propagation and genetic improvement of woody plants, particularly roses.

*Geert-Jan de Kler*k (see chapter 3)

Chapter 5.

Ivana Machackova is a Professor at the Institute of Experimental Botany of the Academy of Sciences of the Czech Republic in Prague (Czech Republic). She is Head of the Laboratory of Plant Morphogenesis and Director of the Institute. She lectures in the Department of Plant Physiology at the Charles University in Prgaue. Her research interests are in the area of plant growth substances (auxins, ethylene, abscisic acid and melatonin); their modes of action and metabolism, regulation of their levels in relation to plant development and electrophysiology.

Eva Zazimalova is an Associate Professor of Plant Physiology at the Institute of Experimental Botany of the Academy of Sciences of the Czech Republic in Prague. She is Head of the Laboratory of Hormonal Regulation in Plants and Deputy Director of the Institute. She also teaches in the Department of Plant Physiology at the Charles University in Prague. Her research is in the fields of auxin and cytokinins (mode of action of auxin, auxin binding site(s), regulation of levels of auxins and cytokinins in relation to cell division and elongation and the mechanism of polar transport of auxin).

Chapter 6.

Johannes van Staden was awarded his PhD (Botany) in 1970 and lectured in this field until 2003. He is a Professor and Director of the Research Institute for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal (South Africa). His main interests are in the hormonal regulation of plant growth, seed germination, plant tissue culture and ethnobotany/medicine.

Eva Zazimalova (see chapter 5).

Chapter 7.

Igor E Moshkov is a Leading Researcher in plant physiology and biochemistry and Deputy Director at the Timiryazev Institute of Plant Physiology, Russian Academy of Science, Moscow. His research is focussed on ethylene signal perception and transduction, the interaction between ethylene and cytokinin at the level of hormone perception and signal transduction pathways and GTP-binding proteins in phytohormone signalling.

Galina V Novikova is a Leading Researcher in plant physiology and biochemistry at the Timiryazev Institute of Plant Physiology, Russian Academy of Science, Moscow. Her research is related to the mode of action of phytohormone action (cytokinins and ethylene) and interactions of the phytohormones, protein phosphorylation/dephosphorylation in relation to phytohormone signal perception and transduction and MAPK cascades in phytohormone signal transduction.

Michael A Hall has been Professor of Botany at the University of Wales, Aberystwyth (UK) since 1981. His research is involved with signal perception and transduction mechanisms for plant hormones, especially ethylene, as well as the role of hormones in the responses of plants to environmental stress.

Chapter 8.

Dominique Chriqui is Professor and Director of a laboratory of plant development at the University Pierre and Marie Curie, Paris (France). She has been involved for many years in research on the cellular and molecular features that underlie morphogenic events such as rhizogenesis and shoot regeneration, both in planta and *in vitro*. She is now particularly interested in the early events of the regenerative process and in the interfaces between hormones, cell cycle and developmental genes and has published approx. 100 papers in the field of plant morphogenesis.

Chapter 9.

Sara von Arnold holds a PhD from Uppsala University (1979), Sweden. She has been a full Professor in the Cell Biology of forest trees at the Swedish University of Agricultural Sciences, Uppsala since 1988. Her research focusses on developmental processes in conifers and especially somatic embryogenesis.

Chapter 10.

Peter B Gahan is Emeritus Professor of Cell Biology at King's College London (UK) with fifty years of research and teaching experience in plant and animal biology. He is interested in the mechanism of competence and recalcitrance of plant cells to regenerate and also in the role of DNA as a messenger between cells and tissues.

Chapter 11.

John Preece is a horticulture professor in the Department of Plant, Soil and Agricultural Systems at Southern Illinois University Carbondale (USA). He teaches courses in General Horticulture, Plant Propagation and Plant Growth and Development. He conducts research on various aspects of woody plant propagation. Along with his postgraduates, he was the first to publish micropropagation protocols for a number of woody species and the first to work out somatic embryogenesis and shoot morphogenesis of *Fraxinus americana* (white ash) and *Juglans nigra* (eastern black walnut).

Chapter 12.

William (Bill) Davies is currently Professor of Environmental Plant Biology at Lancaster University (UK) and Director of the Lancaster Environmental centre, one of the largest groups of environmetal researchers in Europe. He obtained his first degree in Horticultural Science from the University of Reading (UK) and his PhD in Forestry and Botany from the University of Wisconsin, Madison (USA). His research interests include regulation of growth and functioning of plants experiencing environmental stress; stomatal physiology, root to shoot communication via chemical signalling in plants; environmental physiology of crops and native species; crop improvement for water-scarce environments; irrigation science and enhancing the efficiency of crop water use through novel management techniques. He has published more than 200 papers in international plant science journals and edited 17 books. He is a member of the ISI database of 'Highly Cited Researchers' in Plant and Animal Sciences. He is a member of the Defra Horticulture

Link programme Management Committee and editor-in-chief of the *Journal of Experimental Botany*.

Chapter 13.

Meira Ziv is a Professor in the Robert H Smith Institute of Plant Science and Genetics at the Hebrew University of Jerusalem (Israel). Her research interests are in the physiology and morphogenesis of plant organogenesis and somatic embryogenesis in large scale liquid cultures; shoot-malformation, hyperhydricity and the role of oxidative stress in the control of plant development in bioreactor cultures for efficient acclimatization and survival *ex vitro*; bulb and corm development in geophytes cultured in liquid cultures in relation to carbohydrate metabolism.

Jianxin Chen is a research scientist in the Department of Biology at Brock University, Ontario (Canada). His interests are in large-scale micropropagation, metabolic pathways and cloning of medicinal plants and plant breeding.

Chapter 1
Plant Tissue Culture Procedure - Background

1. INTRODUCTION

Plant tissue culture is the science of growing plant cells, tissues or organs isolated from the mother plant, on artificial media. It includes techniques and methods used to research into many botanical disciplines and has several practical objectives. Before beginning to propagate plants by tissue culture methods, it is necessary to have a clear understanding of the ways in which plant material can be grown and manipulated in 'test tubes'. This chapter therefore describes the techniques that have been developed for the isolation and *in vitro* culture of plant material, and shows where further information can be obtained. Both organised and unorganised growth are possible *in vitro*.

1.1. ORGANISED GROWTH

Organised growth contributes towards the creation or maintenance of a defined structure. It occurs when plant organs such as the growing points of shoots or roots (apical meristems), leaf initials, young flower buds or small fruits, are transferred to culture and continue to grow with their structure preserved. Growth that is coherently organised also occurs when organs are induced. This may occur *in vitro* either directly upon an organ or upon a piece of tissue placed in culture (an explant), or during the culture of previously unorganised tissues. The process of *de novo* organ formation is called organogenesis or morphogenesis (the development of form).

1.2. UNORGANISED GROWTH

The growth of higher plants depends on the organised allocation of functions to organs which in consequence become differentiated, that is to say, modified and specialised to enable them undertake their essential roles. Unorganised growth is seldom found in nature, but occurs fairly frequently when pieces of whole plants are cultured *in vitro*. The cell aggregates, which are then formed, typically lack any recognisable structure and contain only a limited number of the many kinds of specialised and differentiated cells found in an intact plant. A differentiated cell is one that has developed a specialised form (morphology) and/or function (physiology). A differentiated tissue (e.g. xylem or epidermis) is an aggregation of differentiated cells. So far, the formation of differentiated cell types can only be controlled to a limited extent in culture. It is not possible, for example, to maintain and multiply a culture composed entirely of epidermal cells. By contrast, unorganised tissues can be increased in volume by subculture and can be maintained on semi-solid or liquid media for long periods. They can often also be used to commence cell suspension cultures. Differentiation is also used botanically to describe the formation of distinct organs through morphogenesis.

2. TISSUE CULTURE

2.1. CULTURES OF ORGANISED STRUCTURES

Organ culture is used as a general term for those types of culture in which an organised form of growth can be continuously maintained. It includes the aseptic isolation from whole plants of such definite structures as leaf primordia, immature flowers and fruits, and their growth *in vitro*. For the purposes of plant propagation, the most important kinds of organ culture are:

- Meristem cultures, in which are grown very small excised shoot apices, each consisting of the apical meristematic dome with or without one or two leaf primordia. The shoot apex is typically grown to give one single shoot.
- Shoot tip, or shoot cultures, started from excised shoot tips, or buds, larger than the shoot apices employed to establish meristem cultures, having several leaf primordia. These shoot apices are usually cultured in such a way that each produces multiple shoots.
- Node cultures of separate lateral buds, each carried on a small piece of stem tissue; stem pieces carrying either single or multiple nodes may be cultured. Each bud is grown to provide a single shoot.
- Isolated root cultures. The growth of roots, unconnected to shoots: a branched root system may be obtained.

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- Embryo cultures, where fertilised or unfertilised zygotic (seed) embryos are dissected out of developing seeds or fruits and cultured *in vitro* until they have grown into seedlings. Embryo culture is quite distinct from somatic embryogenesis (see below).

These types of cultures are described in more detail later in this chapter.

2.2. CULTURES OF UNORGANISED TISSUES

'Tissue culture' is commonly used as a collective term to describe all kinds of *in vitro* plant cultures although strictly it should refer only to cultures of unorganised aggregates of cells. In practice the following kinds of cultures are most generally recognised:

- Callus (or tissue) cultures. The growth and maintenance of largely unorganised cell masses, which arise from the uncoordinated and disorganised growth of small plant organs, pieces of plant tissue, or previously cultured cells.
- Suspension (or cell) cultures. Populations of plant cells and small cell clumps, dispersed in an agitated, that is aerated, liquid medium.
- Protoplast cultures. The culture of plant cells that have been isolated without a cell wall.
- Anther cultures. The culture of complete anthers containing immature pollen microspores. The objective is usually to obtain haploid plants by the formation of somatic embryos (see below) directly from the pollen, or sometimes by organogenesis *via* callus. Pollen cultures are those initiated from pollen that has been removed from anthers.

2.3. USING TISSUE CULTURES FOR PLANT PROPAGATION

The objective of plant propagation via tissue culture, termed micropropagation, is to propagate plants true-to-type, that is, as clones. Plants obtained from tissue culture are called microplants and can be derived from tissue cultures in three ways:

- from pre-existing shoot buds or primordial buds (meristems) which are encouraged to grow and proliferate;
- following shoot morphogenesis when new shoots are induced to form in unorganised tissues or directly upon explanted tissues of the mother plant;
- through the formation of somatic embryos which resemble the seed embryos of intact plants, and which can grow into seedlings in the same way. This process is called somatic embryogenesis.

To obtain plants by the first two of these methods, it is necessary to treat shoots of an adequate size as miniature cuttings and induce them to produce roots.

The derivation of new plants from cells, which would not normally have taken part in the process of regeneration, shows that living, differentiated plant cells may express totipotency, i.e. they each retain a latent capacity to produce a whole plant. Totipotency is a special characteristic of cells in young tissues and meristems. It can be exhibited by some differentiated cells, e.g. cambial cells and leaf palisade cells but not those which have developed into terminally differentiated structures (e.g. sieve tubes or tracheids).

Theoretically, plant cells, organs, or plants, can all be cloned, i.e., produced in large numbers as a population where all the individuals have the same genetic constitution as the parent. Present tissue culture techniques do not permit this in every case and irregularities do sometimes occur, resulting in 'somaclonal variants' (Larkin and Scowcroft, 1981). Nevertheless, as will be described in the chapters, which follow, a very large measure of success can be achieved and cultures of various kinds can be used to propagate plants.

2.4. INITIATING TISSUE CULTURES

2.4.1. Explants

Tissue cultures are started from pieces of whole plants. The small organs or pieces of tissue that are used are called explants. The part of the plant (the stock plant or mother plant) from which explants are obtained, depends on:

- the kind of culture to be initiated;
- the purpose of the proposed culture;
- the plant species to be used.

Explants can therefore be of many different kinds. The correct choice of explant material can have an important effect on the success of tissue culture. Plants growing in the external environment are invariably contaminated with micro-organisms and pests. These contaminants are mainly confined to the outer surfaces of the plant, although, some microbes and viruses may be systemic within the tissues (Cassells, 1997). Because they are started from small explants and must be grown on nutritive media that are also favourable for the growth of micro-organisms, plant tissue cultures must usually be established and maintained in aseptic conditions. Most kinds of microbial organism, and in particular bacteria and fungi, compete adversely with plant material growing *in vitro*. Therefore, as far as

possible, explants must be free from microbial contaminants when they are first placed on a nutrient medium. This usually involves growing stock plants in ways that will minimise infection, treating the plant material with disinfecting chemicals to kill superficial microbes, and sterilising the tools used for dissection and the vessels and media in which cultures are grown (for a review see Cassells and Doyle, 2005). Some kinds of plants can, however, be micropropagated in non-sterile environments (see Chapter 3).

2.4.2. Isolation and incubation

The work of isolating and transferring cultured plant material is usually performed in special rooms or inside hoods or cabinets from which micro-organisms can be excluded. Cabinets used for isolation can be placed in a draught-free part of a general laboratory, but are much better situated in a special inoculation or transfer room reserved for the purpose. The accommodation, equipment and methods that are required for successful inoculation and transfer are described in Volume 2. Cultures, once initiated, are placed in incubators or growth rooms where lighting, temperature and humidity can be controlled. The rate of growth of a culture will depend on the temperature (and sometimes the lighting) regime adopted.

2.4.3. The cultural environment

Plant cultures are commenced by placing one or more explants into a pre-sterilised container of sterile nutrient medium. Some explants may fail to grow, or may die, due to microbial contamination: to ensure the survival of an adequate number, it therefore is usual to initiate several cultures at the same time, each being started from an identical organ or piece of tissue. Explants taken from stock plants at different times of the year may not give reproducible results in tissue culture. This may be due to variation in the level of external contaminants or because of seasonal changes in endogenous (internal) growth regulator levels in the stock plant (see Chapter 11).

2.4.4. Media

Plant material will only grow *in vitro* when provided with specialised media. A medium usually consists of a solution of salts supplying the major and minor elements necessary for the growth of whole plants, together with:

- various vitamins (optional);
- various amino acids (optional);
- an energy source (usually sucrose).

The components of plant tissue culture media are discussed in Chapters 3 and 4. The compositions of specific media are described in Volume 2. Growth and development of plant cultures usually also depends on the addition of plant growth regulators to the medium (see Chapters 5, 6 and 7). Plant growth regulators are compounds, which, at very low concentration, are capable of modifying growth or plant morphogenesis. Many workers define a medium as a completed mixture of nutrients and growth regulators. This is a rather inflexible method, as growth regulators frequently need to be altered according to the variety of plant, or at different stages of culture, whilst the basic medium can stay unchanged. It is therefore recommended that nutritional and regulatory components should be listed separately. Plant material can be cultured either in a liquid medium or on a medium that has been partially solidified with a gelling agent (see Chapter 4). The method employed will depend on the type of culture and its objective.

2.4.5. Solidified media

Media which have had a gelling agent added to them, so that they have become semi-solid, are widely used for explant establishment; they are also employed for much routine culture of callus or plant organs (including micropropagation), and for the long-term maintenance of cultures. Agar is the most common solidifying agent, but a gellan gum is also widely used (Chapter 4).

Cultures grown on solid media are kept static. They require only simple containers of glass or plastic, which occupy little space. Only the lower surface of the explant, organ or tissue is in contact with the medium. This means that as growth proceeds there may be gradients in nutrients, growth factors and the waste products of metabolism, between the medium and the tissues. Gaseous diffusion into and out of the cells at the base of the organ or tissue may also be restricted by the surrounding medium.

2.4.6. Liquid media

Liquid media are essential for suspension cultures, and are preferred for critical experiments on the nutrition, growth and cell differentiation in callus tissues. They are also used in some micropropagation work. Very small organs (e.g. anthers) are often floated on the top of liquid medium and plant cells or protoplasts can be cultured in very shallow layers of

static liquid, providing there is sufficient gaseous diffusion. Larger organs such as shoots (e.g. proliferating shoots of shoot cultures) can also often be grown satisfactorily in a shallow layer of non-agitated liquid where part of the organ protrudes above the surface. However, some method of support is necessary for small organs or small pieces of tissue, which would otherwise sink below the surface of a static liquid medium, or they will die for lack of aeration. Systems of support which have been found to be effective and which can be used instead of agar-solidified media are described in Chapters 4.

Many tissues and organs, small and large, also grow well unsupported in a liquid medium, providing it is aerated by shaking or moving (see below). Some kind of agitation is essential for suspension cultures to prevent cells and cell aggregates settling to the bottom of the flask. Other purposes served by agitation include: the provision of increased aeration, the reduction of plant polarity, the uniform distribution of nutrients and the dilution of toxic explant exudates (Lim-Ho, 1982).

There are several alternative techniques. Plant cell suspensions can be cultured very satisfactorily when totally immersed in a liquid culture medium, providing it is shaken (by a rotary or reciprocating shaking machine) or stirred (e.g. by a magnetic stirrer) to ensure adequate aeration. This method may also be used for culturing organs of some plants (e.g. proliferating shoot cultures), but the fragmentation, which occurs, can be disadvantageous.

Periodic immersion may be achieved by growing cultured material in tubes or flasks of liquid medium which are rotated slowly. Steward and Shantz (1956) devised so-called 'nipple flasks' for this purpose which had several side-arms. They were fixed to a wooden wheel, which was rotated so that tissue in the arms of each flask was alternately bathed in medium and drained or exposed to the air (Fig. 1.1). This technique ensured that callus tissue for which they were used was well aerated. The medium usually became turbid as cells dissociated from the callus and started a cell suspension. Flasks of this sort are seldom used to-day because of their cost. A similar alternating exposure can be achieved by placing calluses in vessels, which are rotated slowly.

An alternative to the costly rotating systems to achieve periodic immersion of the cultures, is the increasingly popular temporary immersion system in which static vessels are periodically or temporarily flooded with culture medium (Fig. 1.2; Teisson and Alvard, 1995). Medium is pumped from a reservoir container into the culture vessel for experimentally determined time intervals repeated over a 24 hour cycle. This system prevents anoxia and has the advantage that the medium can easily be changed in the reservoir.

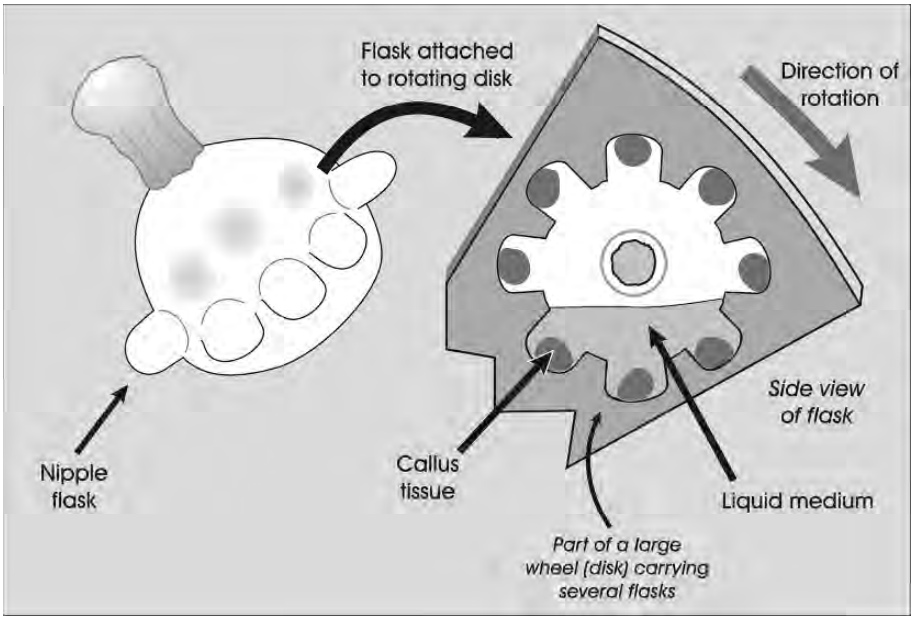

Fig. 1.1 A nipple flask for growing callus in a liquid medium.

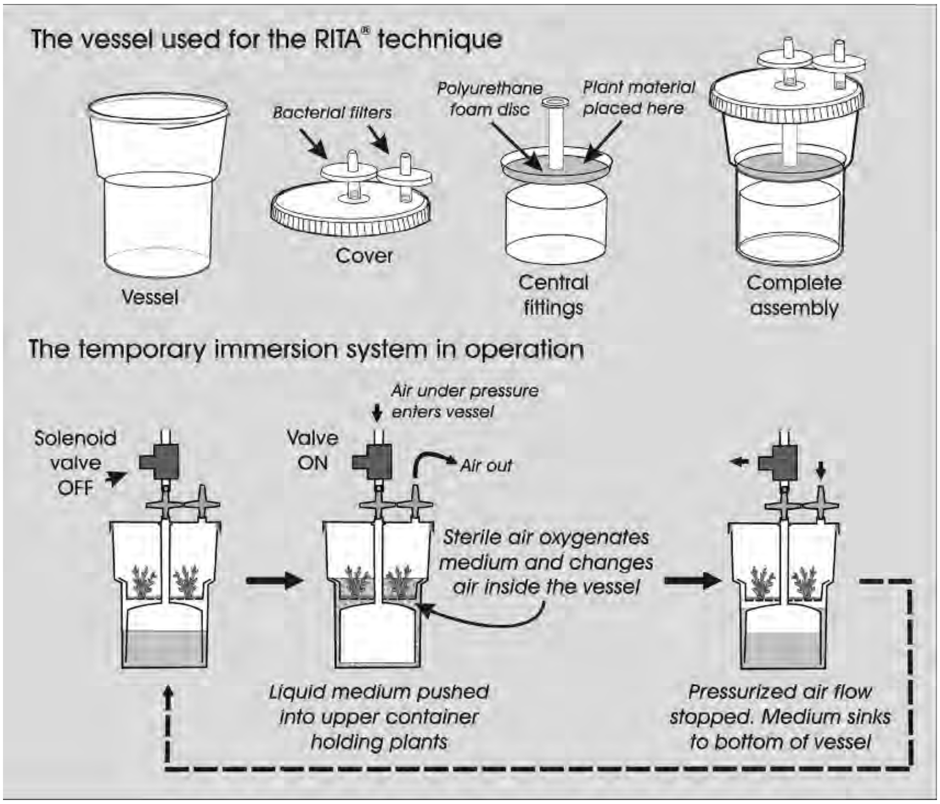

Fig. 1.2 An illustration of the RITA ebb and flow culture vessel.

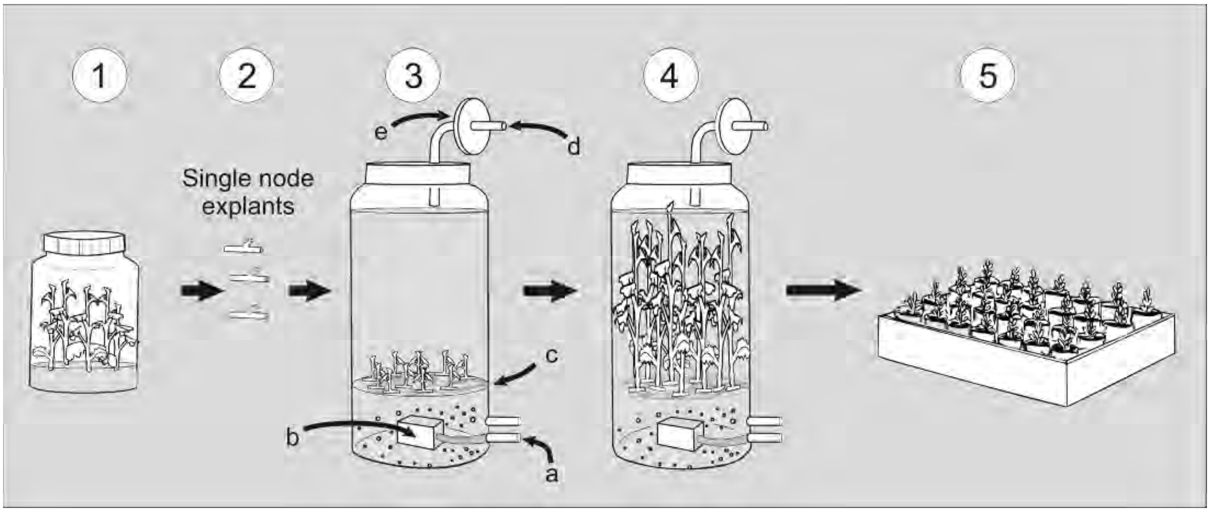

Fig. 1.3 An illustration of micropropagation in a bioreactor.
a. air inlet; b. sparger; c. raft supporting explants; d. air outlet; e. membrane filter. The bioreactor culture is initiated by inoculation with nodes or buds from conventional agar culture. For details of bioreactor design see Fig. 1.9.

Liquid medium in flasks or column bio-reactors (fermentors) can be circulated and at the same time aerated, by the introduction of sterile air. Shearing forces within air-lift reactors are much less than in mechanically-stirred vessels so that plant cell suspensions suffer less damage. Bio-reactors are

used in the pharmaceutical industry to produce high value plant secondary products and to carry out substrate conversions. Low cost bio-reactors developed for micropropagation have been described in detail in Hvoslef-Eide and Preil (2005) (Fig. 1.3).

Rather than immersing callus or organ cultures, liquid medium may be slowly dripped onto the growing tissues or applied as a mist and afterwards the liquid drained or pumped away for recirculation (Weathers and Giles, 1987). A particular advantage of this technique is the ability to grow cultures in a constant and non-depleted medium; nutrients can be varied frequently and rapidly and their availability controlled by altering either concentration or flow rate. Toxic metabolites, which in a closed container might accumulate and inhibit growth, can be removed continuously. As complicated apparatus is needed, the method has not been widely used.

The relative merits of solid and liquid media (and combinations of both) are discussed further in Chapter 12.

2.5. PROBLEMS OF ESTABLISHMENT

2.5.1. Phenolic oxidation

Some plants, particularly tropical species, contain high concentrations of phenolic substances that are oxidised when cells are wounded or senescent. Isolated tissue then becomes brown or black and fails to grow. The prevention of blackening, which can be a serious problem in tissue culture, is discussed in Chapter 11.

2.5.2. Minimum inoculation density

Certain essential substances can pass out of plant cells by diffusion. Substances known to be released into the medium by this means include alkaloids, amino acids, enzymes, growth substances and vitamins (Street, 1969). The loss is of no consequence when there is a large cluster of cells growing in close proximity or where the ratio of plant material to medium is high. However, when cells are inoculated onto an ordinary growth medium at a low population density, the concentration of essential substances in the cells and in the medium can become inadequate for the survival of the culture. For successful culture initiation, there is thus a minimum size of explant or quantity of separated cells or protoplasts per unit culture volume. Inoculation density also affects the initial rate of growth *in vitro*. Large explants generally survive more frequently and grow more rapidly at the outset than very small ones. In practice, minimum inoculation density varies according to the genotype of plant being cultured and the cultural conditions. For commencing suspension cultures it is commonly about 1-1.5 x 10^4 cells/ml.

The minimum cell density phenomenon is sometimes called a ‘feeder effect’ because deficiencies can often be made up by the presence of other cells growing nearby. Suspension cultures can be started from a low density of inoculum by ‘conditioning’ a freshly prepared medium - i.e. allowing products to diffuse into it from a medium in which another culture is growing actively, or adding a quantity of filter-sterilised medium which has previously supported another culture. The use of conditioned media can reduce the critical initial cell density by a factor of about 10 (Stuart and Street, 1969).

It is possible to overcome the deficiencies of plant cells at low starting densities by adding small amounts of known chemicals to a medium. For example, Kao and Michayluk (1975) have shown that *Vicia hajastana* cells or protoplasts can be cultured from very small initial inocula or even from individual cells: a standard culture medium was supplemented with growth regulators, several organic acids, additional sugars (apart from sucrose and glucose), and in particular, casein hydrolysate (casamino acids) and coconut milk.

There is often a maximum as well as a minimum plating or inoculation density for plant cells or protoplasts. In a few cases the effective range has been found to be quite narrow. Some effects of inoculation density on morphogenesis are described in Chapter 10.

2.6. PATTERNS OF GROWTH AND DIFFERENTIATION

A typical unorganised plant callus, initiated from a new explant or a piece of a previously-established culture, has three stages of development, namely:

- the induction of cell division;
- a period of active cell division during which differentiated cells lose any specialised features they may have acquired and become dedifferentiated;
- a period when cell division slows down or ceases and when, within the callus, there is increasing cellular differentiation.

These phases are similarly reproduced by cell suspensions grown in a finite volume of medium (a batch culture), where according to a variety of different parameters that can be used to measure growth (e.g. cell number, cell dry weight, total DNA

content) an S-shaped growth curve is generally obtained (Fig. 1.4).

The phases are:

- a lag phase;
- a period of exponential and then linear growth;
- a period when the rate of growth declines;
- a stationary phase when growth comes to a halt.

Some differentiation of cells may occur in cell cultures during the period of slowed and stationary growth, but generally it is less marked and less complete than that which occurs in callus cultures. Cultures cannot be maintained in stationary phase for long periods. Cells begin to die and, as their contents enter the nutrient medium, death of the whole culture accelerates. Somewhat similar patterns of growth also occur in cultures of organised structures. These also cease growth and become moribund as the components of the medium become exhausted.

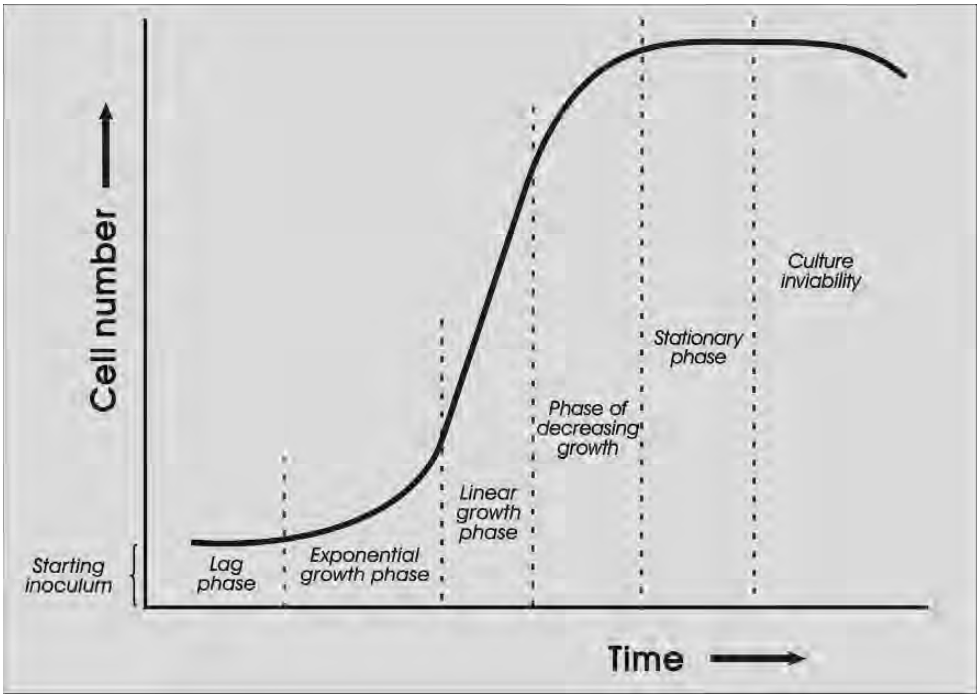

Fig. 1.4 Diagram showing the phases of growth in batch suspension culture.

2.7. SUBCULTURING

Once a particular kind of organised or unorganised growth has been started *in vitro*, it will usually continue if callus cultures, suspension cultures, or cultures of indeterminate organs (see below) are divided to provide new explants for culture initiation on fresh medium. Subculturing often becomes imperative when the density of cells, tissue or organs becomes excessive; to increase the volume of a culture; or to increase the number of organs (e.g. shoots or somatic embryos) for micropropagation. The period from the initiation of a culture or a subculture to the time of its transfer is sometimes called a passage. The first passage is that in which the original explant or inoculum is introduced.

Suspensions regularly subcultured at the end of the period of exponential growth can often be propagated over many passages. However, many cultures reach a peak of cell aggregation at this time and aggregation often becomes progressively more pronounced in subsequent passages (Street, 1977b). Subculture is therefore more conveniently carried out during the stationary phase when cell aggregation is least pronounced. Rapid rates of plant propagation depend on the ability to subculture shoots from proliferating shoot or node cultures, from cultures giving direct shoot regeneration, or callus or suspensions capable of reliable shoot or embryo regeneration.

A further reason for transfer, or subculture, is that the growth of plant material in a closed vessel eventually leads to the accumulation of toxic metabolites and the exhaustion of the medium, or to its drying out. Thus, even to maintain the culture, all or part of it must be transferred onto fresh medium. Callus subcultures are usually initiated by moving a fragment of the initial callus (an inoculum) to fresh medium in another vessel. Shoot cultures are subcultured by segmenting individual shoots or shoot clusters. The interval between subcultures depends on the rate at which a culture has grown: at 25°C, subculturing is typically required every 4-6 weeks. In the early stages of callus growth it may be convenient to transfer the whole piece of tissue to fresh medium, but a more established culture will need to be divided and only small selected portions used as inocula. Regrowth depends on the transfer of healthy tissues.

Decontamination procedures are theoretically no longer necessary during subculturing, although sterile transfer procedures must still be used. However, when using shoot or node cultures for micropropagation, some laboratories do re-sterilise plant material at this stage as a precaution against the spread of contaminants (see Volume 2). Cultures which are obviously infected with micro-organisms should not be used for subculturing and should be autoclaved before disposal.

2.8. SUBCULTURING HAZARDS

There are several hazards in subculturing which are discussed more fully in other chapters of this book. Several kinds of callus may arise from the initial explant, each with different morphogenic potential. Strains of callus tissue capable of giving rise to somatic embryos and others without this capability can, for instance, arise simultaneously from the culture of grass and cereal seed embryos. Careful selection of the correct strain is therefore necessary if cultures capable of producing somatic embryos are ultimately required. Timing of the transfer may also be important, because if left alone for some while, non-embryogenic callus may grow from the original explant at the expense of the competent tissue, which will then be obscured or lost.

Although subculturing can often be continued over many months without adverse effects becoming apparent, cultures of most unorganised cells and of some organised structures can accumulate cells that are genetically changed. This may cause the characteristics of the culture to be altered and may mean that some of the plants regenerated from the culture will not be the same as the parent plant. This subject is discussed further in Chapter 2. Cultures may also inexplicably decline in vigour after a number of passages, so that further subculture becomes impossible.

3. TYPES OF TISSUE CULTURE

3.1. ORGAN CULTURES

Differentiated plant organs can usually be grown in culture without loss of integrity. They can be of two types:

- Determinate organs which are destined to have only a defined size and shape (e.g. leaves, flowers and fruits);
- Indeterminate organs, where growth is potentially unlimited (apical meristems of roots and non-flowering shoots).

In the past, it has been thought that the meristematic cells within root or shoot apices were not committed to a particular kind of development. It is now accepted that, like the primordia of determinate organs such as leaves, apical meristems also become inherently programmed (or determined) into either root or shoot pathways (see Chapter 8). The eventual pattern of development of both indeterminate and determinate organs is often established at a very early stage. For example, the meristematic protrusions in a shoot apex become programmed to develop as either lateral buds or leaves after only a few cell divisions have taken place (see Chapter 10).

3.1.1. Culture of determinate organs

An organ arises from a group of meristematic cells. In an indeterminate organ, such cells are theoretically able to continue in the same pattern of growth indefinitely. The situation is different in the primordium of a determinate organ. Here, as meristematic cells receive instructions on how to differentiate, their capacity for further division becomes limited.

If the primordium of a determinate organ is excised and transferred to culture, it will sometimes continue to grow to maturity. The organ obtained *in vitro* may be smaller than that which would have developed on the original plant *in vivo*, but otherwise is likely to be normal. The growth of determinate organs cannot be extended by subculture as growth ceases when they have reached their maximum size.

Organs of limited growth potential, which have been cultured, include leaves (Caponetti and Steeves, 1963; Caponetti, 1972); fruits (Nitsch, 1951, 1963; Street, 1969); stamens (Rastogi and Sawhney, 1988); ovaries and ovules (which develop and grow into embryos) and flower buds of several dicotyledonous plant species (Table 1.1).

Until recently, a completely normal development was obtained in only a few cases. This was probably due to the use of media of sub-optimum composition. By experimenting with media constituents, Berghoef and Bruinsma (1979a) obtained normal growth of *Begonia franconis* buds and were thus able to study the effect of plant growth substances and nutritional factors on flower development and sexual expression (Berghoef and Bruinsma, 1979b). Similarly, by culturing dormant buds of *Salix*, Angrish and Nanda (1982a,b) could study the effect of bud position and the progressive influence of a resting period on the determination of meristems to become catkins and fertile flowers. In several species, flowers have been pollinated *in vitro* and have then given rise to mature fruits (e.g. Ruddat *et al.,* 1979)

Table 1.1 Some species in which flower buds have been cultured

Cucumis sativus	Galun *et al.* (1962)
Viscaria spp.	Blake (1966, 1969)
Nicotiana tabacum	Hicks and Sussex (1970)
Aquilegia formosa	Bilderback (1971)
Cleome iberidella	De Jong and Bruinsma (1974)
Nicotiana offinis	Deaton *et al.* (1980)

Plants cannot be propagated by culturing meristems already committed to produce determinate organs, but providing development has not proceeded too far, flower meristems can often be induced to revert to vegetative meristems *in vitro*. In some plants the production of vegetative shoots from the flower meristems on a large inflorescence can provide a convenient method of micropropagation (see Chapter 2).

3.1.2. Culture of indeterminate organs

Meristem and shoot culture. The growing points of shoots can be cultured in such a way that they continue uninterrupted and organised growth. As these shoot initials ultimately give rise to small organised shoots which can then be rooted, their culture has great practical significance for plant propagation. Two important uses have emerged:

Meristem culture. Culture of the extreme tip of the shoot, is used as a technique to free plants from virus infections. Explants are dissected from either apical or lateral buds. They comprise a very small stem apex (0.2-1.0 mm in length) consisting of just the apical meristem and one or two leaf primordia;

Shoot culture or shoot tip culture. Culture of larger stem apices or lateral buds (ranging from 5 or 10 mm in length to undissected buds) is used as a very successful method of propagating plants.

The size and relative positions of the two kinds of explant in a shoot apex of a typical dicotyledon is shown in Fig. 1.5. Node culture is an adaptation of shoot culture.

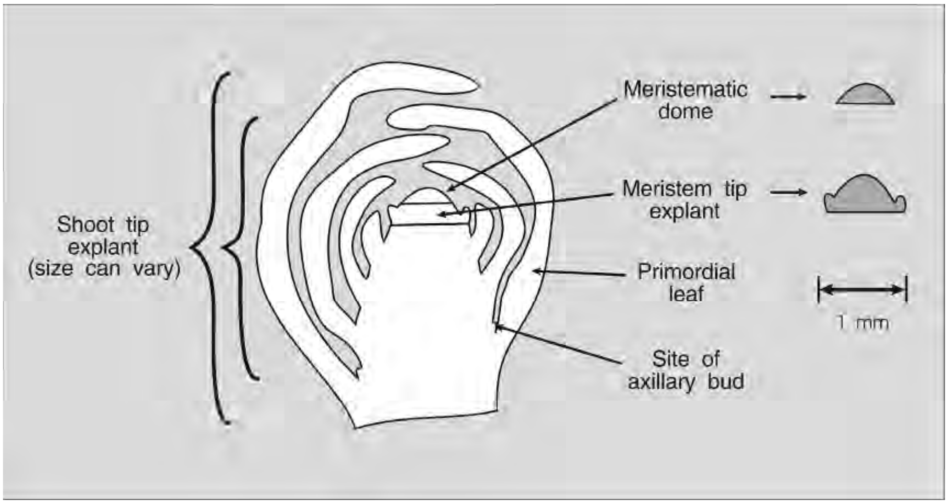

Fig. 1.5 A diagrammatic section through a bud showing the locations and approximate relative sizes of a meristematic dome, the meristem tip and shoot tip explants.

If successful, meristem culture, shoot culture and node culture can ultimately result in the growth of small shoots. With appropriate treatments, these original shoots can either be rooted to produce small plants or 'plantlets', or their axillary buds can be induced to grow to form a cluster of shoots. Plants are propagated by dividing and reculturing the shoot clusters, or by growing individual shoots for subdivision. At a chosen stage, individual shoots or shoot clusters are rooted. Tissue cultured shoots are removed from aseptic conditions at or just before the rooting stage, and rooted plantlets are hardened off and grown normally. Shoot culture, node culture and meristem tip culture are discussed in greater detail in Chapter 2.

Embryo culture. Zygotic or seed embryos are often used advantageously as explants in plant tissue culture, for example, to initiate callus cultures. In embryo culture however, embryos are dissected from seeds, individually isolated and 'germinated' *in vitro* to provide one plant per explant. Isolated embryo culture can assist in the rapid production of seedlings from seeds that have a protracted dormancy period, and it enables seedlings to be produced when the genotype (e.g. that resulting from some interspecific crosses) conveys a low embryo or seed viability.

During the course of evolution, natural incompatibility systems have developed which limit the types of possible sexual crosses (see De Nettancourt and Devreux, 1977). Two kinds of infertility occur:

- Pre-zygotic incompatibility, preventing pollen germination and/or pollen tube growth so that a zygote is never formed;
- Post-zygotic incompatibility, in which a zygote is produced but not accepted by the endosperm. The embryo, not receiving sufficient nutrition, disintegrates or aborts.

Pre-zygotic incompatibility can sometimes be overcome in the laboratory using a technique developed by Kanta *et al.* (1962) called *in vitro* pollination (or *in vitro* fertilisation). For a description of this technique see review articles by Ranga Swamy (1977), Zenkteler (1980) and Yeung *et al.* (1981). Reviews of embryo culture have been provided by Torrey (1973), Norstog (1979) and Raghavan (1967, 1977a, 1980).

Embryo culture has been used successfully in a large number of plant genera to overcome post-zygotic incompatibility which otherwise hampers the production of desirable hybrid seedlings. For example, in trying to transfer insect resistance from a wild *Solanum* species into the aubergine, Sharma *et al.* (1980a) obtained a few hybrid plants (*Solanum melongena* x *S. khasianum*) by embryo culture. Embryo culture in these circumstances is more aptly termed embryo rescue. Success rates are usually quite low and the new hybrids, particularly if they arise from remote crosses, are sometimes sterile. However, this does not matter if the plants can afterwards be propagated asexually. Hybrids between incompatible varieties of tree and soft fruits (Tukey, 1934; Skirm, 1942) and *Iris* (in Reuther, 1977) have been obtained by culturing fairly mature embryos.

Fruits or seeds are surface sterilised before embryo removal. Providing aseptic techniques are strictly adhered to during excision and transfer to a culture medium, the embryo itself needs no further sterilisation. To ease the dissection of the embryo, hard seeds are soaked in water to soften them, but if softening takes more than a few hours it is advisable to re-sterilise the seed afterwards. A dissecting microscope may be necessary to excise the embryos from small seeds as it is particularly important that the embryo should not be damaged.

Culture of immature embryos (pro-embryos) a few days after pollination frequently results in a greater proportion of seedlings being obtained than if more mature embryos are used as explants, because incompatibility mechanisms have less time to take effect. Unfortunately dissection of very small embryos requires much skill and cannot be done rapidly: it also frequently results in damage which prevents growth *in vitro*. In soybean, Hu and Sussex (1986) obtained the best *in vitro* growth of immature embryos if they were isolated with their suspensors intact. Excised embryos usually develop into seedlings precociously (i.e. before they have reached the size they would have attained in a normal seed).

As an alternative to embryo culture, in some plants it has been possible to excise and culture pollinated ovaries and immature ovules. Ovule culture, sometimes called '*in ovulo* embryo culture', can be more successful than the culture of young embryos. Pro-embryos generally require a complex medium for growth, but embryos contained within the ovule require less complicated media. They are also easily removed from the plant and relatively insensitive to the physical conditions of culture (Thengane *et al.,* 1986). The difference

between embryo and ovule culture is shown diagrammatically in Fig. 1.6.

Because seedlings, which resulted from ovule culture of a *Nicotiana* interspecific cross all died after they had developed some true leaves, Iwai *et al.* (1985) used leaves of the immature seedlings as explants for the initiation of callus cultures. Most shoots regenerated from the callus also died at an early stage, but one gave rise to a plant, which was discovered later to be a sterile hybrid. Plants were also regenerated from callus of a *Pelargonium* hybrid by Kato and Tokumasu (1983). The callus in this case arose directly from globular or heart-shaped zygotic embryos which were not able to grow into seedlings.

The seeds of orchids have neither functional storage organs, nor a true seed coat, so dissection of the embryo would not be possible. In fact, for commercial purposes, orchid seeds are now almost always germinated *in vitro*, and growth is often facilitated by taking immature seeds from green pods (see Volume 2).

Many media have been especially developed for embryo culture and some were the forerunners of the media now used for general tissue culture. Commonly, mature embryos require only inorganic salts supplemented with sucrose, whereas immature embryos have an additional requirement for vitamins, amino acids, growth regulators and sometimes coconut milk or some other endosperm extract. Raghavan (1977b) encouraged the incorporation of mannitol to replace the high osmotic pressure exerted on proembryos by ovular sap. Seedlings obtained from embryos grown *in vitro* are planted out and hardened off in the same manner as other plantlets raised by tissue culture (Chapter 2 and Volume 2).

Although embryo culture is especially useful for plant breeders, it does not lead to the rapid and large scale rates of propagation characteristic of other micropropagation techniques, and so it is not considered further in this book. More details can be found in papers by: Sanders and Ziebur (1958); Raghavan (1967, 1980); Torrey (1973); Zilis and Meyer (1976); Collins and Grosser (1984), Monnier (1990) and Ramming (1990). Yeung *et al.* (1981) have suggested a basic protocol, which with modifications, should be applicable to any species.

The induction of multiple shoots from seeds is described in Chapter 2.

Isolated root culture. Root cultures can be established from root tips taken from primary or lateral roots of many plants. Suitable explants are small sections of roots bearing a primary or lateral root meristem. These explants may be obtained, for example, from surface sterilised seeds germinated in aseptic conditions. If the small root meristems continue normal growth on a suitable medium, they produce a root system consisting only of primary and lateral roots (Fig. 1.7.). No organised shoot buds will be formed.

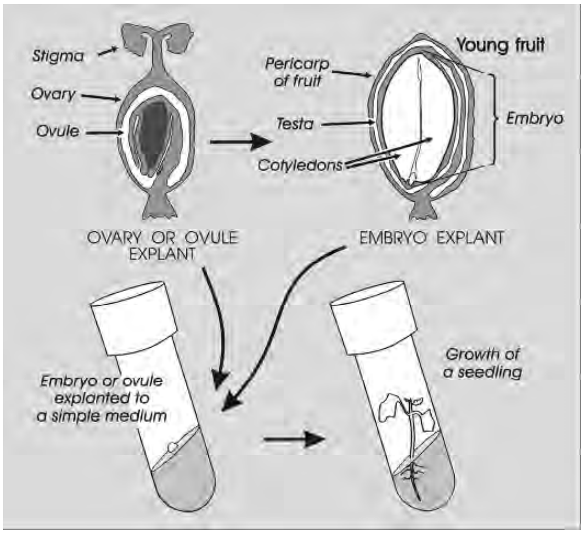

Fig. 1.6 Ovule and embryo culture.

The discovery that roots could be grown apart from shoot tissue was one of the first significant developments of modern tissue culture science. Root culture initially attracted a great deal of attention from research workers and the roots of many different species of plants were cultured successfully (see the comprehensive reviews of Street, 1954, 1957, 1969; and Butcher and Street, 1964).

Plants fall generally into three categories with regard to the ease with which their roots can be cultured. There are some species such as clover, *Datura*, tomato and *Citrus*, where isolated roots can be grown for long periods of time, some seemingly, indefinitely (Said and Murashige, 1979) providing regular subcultures are made. In many woody species, roots have not been grown at all successfully in isolated cultures. In other species such as pea, flax and wheat, roots can be cultured for long periods but ultimately growth declines or insufficient lateral roots are produced to provide explants for subculture.

The inability to maintain isolated root cultures is due to an induced meristematic dormancy or 'senescence', related to the length of time that the

roots have been growing *in vitro*. Transferring dormant meristems to fresh medium does not promote regrowth, possibly due to the accumulation of naturally-occurring auxinic growth substances at the root apex. The addition of so-called anti-auxin, or cytokinin growth regulators can often prolong active growth of root cultures, whereas placing auxins or gibberellic acid in the growth medium, causes it to cease more rapidly. Cultures, which cannot be maintained by transferring root apices, can sometimes be continued if newly-initiated lateral root meristems are used as secondary explants instead.

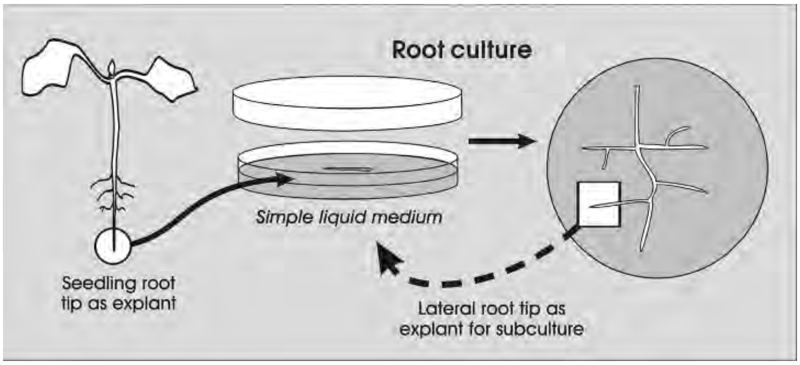

Fig. 1.7 Methods of root culture.

Isolated plant roots can usually be cultured on relatively simple media such as White (1954) containing 2% sucrose. Liquid media are preferable, as growth in or on a solid medium is slower. This is presumably because salts are less readily available to the roots from a solidified medium and oxygen availability may be restricted. Although roots will accept a mixed nitrate/ammonium source, they will not usually grow on ammonium nitrogen alone. Species, and even varieties or strains, of plants, are found to differ in their requirement for growth regulators, particularly for auxins, in the root culture medium.

Isolated root cultures have been employed for a number of different research purposes. They have been particularly valuable in the study of nematode infections and provide a method by which these parasites can be cultured in aseptic conditions. Root cultures may also be used to grow beneficial mycorrhizal fungi, and to study the process of root nodulation with nitrogen-fixing *Rhizobium* bacteria in leguminous plants. For the latter purpose, various special adaptations of standard techniques have been adopted to allow roots to become established in a nitrate-free medium (Raggio *et al.,* 1957; Torrey, 1963).

Unlike some other cultured tissues, root cultures exhibit a high degree of genetic stability (see Chapter 10). It has therefore been suggested that root cultures could afford one means of storing the germplasm of certain species (see Volume 2). For suitable species, root cultures can provide a convenient source of explant material for the micropropagation of plants, but they will only be useful in micropropagation if shoots can be regenerated from roots. There are however, several ways in which this can be done, although they are likely to be effective in only a small number of plant genera which have a natural tendency to produce suckers, or new shoots from whole or severed roots:

- From direct adventitious shoots;
- From shoots or embryos originating indirectly on root callus;
- By conversion of the apical root meristem to a shoot meristem.

Adventitious shoots form readily on the severed roots of some plant species, and root cuttings are employed by horticulturists to increase plants *in vivo* (see, for example, the review by Hodge, 1986). Shoot regeneration from roots has not been widely used as a method of micropropagation, even though direct shoot regeneration from roots has been observed *in vitro* on many plants. Sections of fleshy roots used as primary explants are especially likely to form new shoots. Adventitious shoots always develop at the proximal end of a root section while, as a rule, new roots are produced from the distal end. Isolated root cultures would be useful in micropropagation if shoots could be induced to form directly upon them. Unfortunately plants seem to have a high degree of genetic specificity in their

capacity to produce shoots directly on isolated root cultures. Shoot induction often occurs after the addition of a cytokinin to the medium. Seeliger (1956) obtained shoot buds on cultured roots of *Robinia pseudoacacia* and Torrey (1958), shoot buds on root cultures of *Convolvulus*. Direct shoot formation was induced in three species of *Nicotiana* and on *Solanum melongena* by Zelcer *et al.* (1983) but in *N. tabacum* and *N. petunoides* shoots were only obtained after callus formed on the roots. The most optimistic report we have seen comes from Mudge *et al.* (1986), who thought that the shoot formation, which they could induce in raspberry root cultures would provide a convenient and labour-saving method of multiplying this plant *in vitro*.

Plants may also be regenerated from root-derived callus of some species e.g. tomato (Norton and Boll, 1954); *Isatis tinctoria* (Danckwardt-Lilliestrom, 1957); *Atropa belladonna* (Thomas and Street, 1972). Embryogenesis, leading to the formation of protocorm-like bodies, occurs in the callus derived from the root tips of certain orchids e.g. *Catasetum trulla* x *Catasetum* (Kerbauy, 1984a); *Epidendrum obrienianum* (Stewart and Button, 1978); *Oncidium varicosum* (Kerbauy, 1984b).

Changing the determined nature of a root meristem, so that it is induced to produce a shoot instead of a root, is a very rare event but has been noted to occur *in vitro* in the orchid *Vanilla planifolia*. The quiescent centre of cultured root tip meristems was changed into a shoot meristem so that cultured root tips grew to produce plantlets or multiple shoots (Philip and Nainar, 1986). Ballade (1971) maintained that newly initiated root initials, arising from single nodes of *Nasturtium officinale*, could be made to develop into shoot meristems by placing a crystal of kinetin on each explant which was then transferred to a medium containing 0.05% glucose.

3.2. CULTURE OF UNORGANISED CELLS

3.2.1. Callus cultures

Callus is a coherent and amorphous tissue, formed when plant cells multiply in a disorganised way. It is often induced in or upon parts of an intact plant by wounding, by the presence of insects or micro-organisms, or as a result of stress. Callus can be initiated *in vitro* by placing small pieces of the whole plant (explants) onto a growth-supporting medium under sterile conditions. Under the stimulus of endogenous growth regulators or growth regulating chemicals added to the medium, the metabolism of cells, which were in a quiescent state, is changed, and they begin active division. During this process, cell differentiation and specialisation, which may have been occurring in the intact plant, are reversed, and the explant gives rise to new tissue, which is composed of meristematic and unspecialised cell types.

During dedifferentiation, storage products typically found in resting cells tend to disappear. New meristems are formed in the tissue and these give rise to undifferentiated parenchymatous cells without any of the structural order that was characteristic of the organ or tissue from which they were derived. Although callus remains unorganised, as growth proceeds, some kinds of specialised cells may again be formed. Such differentiation can appear to take place at random, but may be associated with centres of morphogenesis, which can give rise to organs such as roots, shoots and embryos. The *de novo* production of plants from unorganised cultures is often referred to as plant regeneration.

Although most experiments have been conducted with the tissues of higher plants, callus cultures can be established from gymnosperms, ferns, mosses and thallophytes. Many parts of a whole plant may have an ultimate potential to proliferate *in vitro*, but it is frequently found that callus cultures are more easily established from some organs than others. Young meristematic tissues are most suitable, but meristematic areas in older parts of a plant, such as the cambium, can give rise to callus. The choice of tissues from which cultures can be started is greatest in dicotyledonous species. A difference in the capacity of tissue to give rise to callus is particularly apparent in monocotyledons. In most cereals, for example, callus growth can only be obtained from organs such as zygotic embryos, germinating seeds, seed endosperm or the seedling mesocotyl, and very young leaves or leaf sheaths, but so far never from mature leaf tissue (e.g. Green and Phillips, 1975; Dunstan *et al.,* 1978). In sugar cane, callus cultures can only be started from young leaves or leaf bases, not from semi-mature or mature leaf blades.

Even closely associated tissues within one organ may have different potentials for callus origination. Thus when embryos of *Hordeum distichum* at an early stage of differentiation are removed from developing seeds and placed in culture, callus proliferation originates from meristematic mesocotyl cells rather than from the closely adjacent cells of the scutellum and coleorhiza (Granatek and Cockerline, 1979).

The callus formed on an original explant is called 'primary callus'. Secondary callus cultures are initiated from pieces of tissue dissected from primary callus (Fig. 1.8.). Subculture can then often be continued over many years, but the longer callus is maintained, the greater is the risk that the cells thereof will suffer genetic change (see Chapter 10).

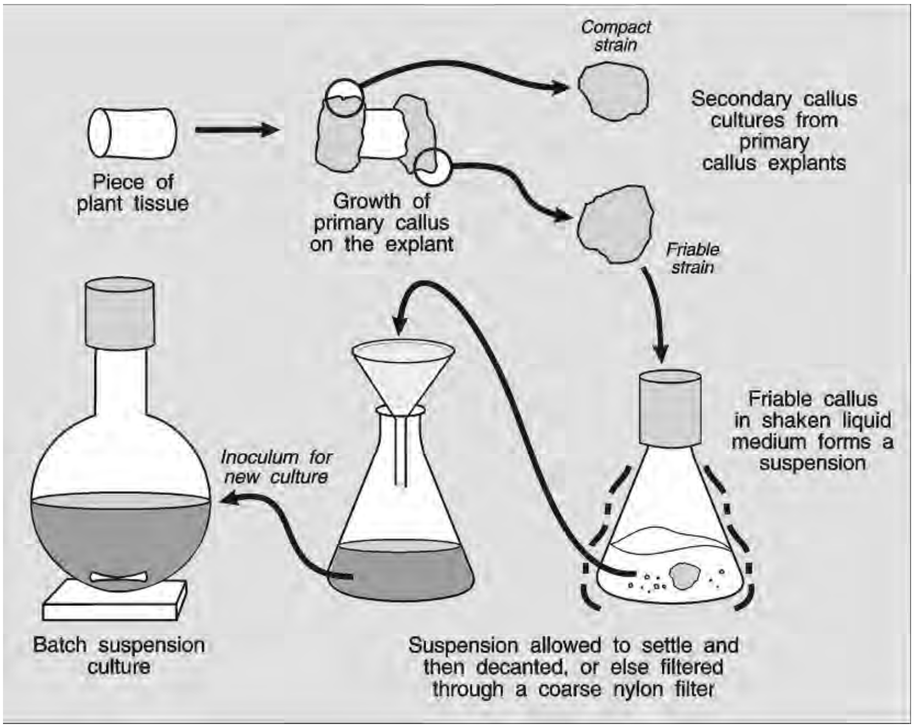

Fig. 1.8 Typical steps in the initiation of callus and suspension cultures.

Callus tissue is not of one single kind. Strains of callus differing in appearance, colour, degree of compaction and morphogenetic potential commonly arise from a single explant. Sometimes the type of callus obtained, its degree of cellular differentiation and its capacity to regenerate new plants, depend upon the origin and age of the tissue chosen as an explant. Loosely packed or 'friable' callus is usually selected for initiating suspension cultures (see below).

Some of the differences between one strain of callus tissue and another can depend on which genetic programme is functioning within the cells (epigenetic differences). Variability is more likely when callus is derived from an explant composed of more than one kind of cell. For this reason there is often merit in selecting small explants from only morphologically uniform tissue, bearing in mind that a minimum size of explant is normally required to obtain callus formation.

The genetic make up of cells is very commonly altered in unorganised callus and suspension cultures. Therefore another reason for cell strains having different characteristics, is that they have become composed of populations of cells with slightly different genotypes. Genetic and epigenetic changes occurring in cultures are described in greater detail in Chapters 10 and Volume 2. The growth, structure, organisation and cytology of callus are discussed in various chapters of the book edited by Street (1977a), and also in the review by Yeoman and Forche (1980).

3.2.2. Cell suspension cultures

Unorganised plant cells can be grown as callus in aggregated tissue masses, or they can be freely dispersed in agitated liquid media. Techniques are similar to those used for the large-scale culture of bacteria. Cell or suspension cultures, as they are called, are usually started by placing an inoculum of friable callus in a liquid medium (Fig. 1.8). Under agitation, single cells break off and, by division, form

cell chains and clumps which fracture again to give individual cells and other small cell groups. It is not always necessary to have a previous callus phase before initiating suspension cultures. For example, leaf sections of *Chenopodium rubrum* floated on Murashige and Skoog (1962) medium in the light, show rapid growth and cell division in the mesophyll, and after 4 days on a rotary shaker they can be disintegrated completely to release a great number of cells into suspension (Geile and Wagner, 1980).

Because the walls of plant cells have a natural tendency to adhere, it is not possible to obtain suspensions that consist only of dispersed single cells. Some progress has been made in selecting cell lines with increased cell separation, but cultures of completely isolated cells have yet to be obtained. The proportion and size of small cell aggregates varies according to plant variety and the medium in which the culture is grown. As cells tend to divide more frequently in aggregates than in isolation, the size of cell clusters increases during the phase of rapid cell division. Because agitation causes single cells, and small groups of cells, to be detached, the size of cell clusters decreases in batch cultures as they approach a stationary growth phase (see below).

The degree of cell dispersion in suspension cultures is particularly influenced by the concentration of growth regulators in the culture medium. Auxinic growth regulators increase the specific activity of enzymes, which bring about the dissolution of the middle lamella of plant cell walls (Torrey and Reinert, 1961). Thus by using a relatively high concentration of an auxin and a low concentration of a cytokinin growth regulator in the culture medium, it is usually possible to increase cell dispersion (Narayanaswamy, 1977). However, the use of high auxin levels to obtain maximum cell dispersion will ensure that the cultured cells remain undifferentiated. This may be a disadvantage if a suspension is being used to produce secondary metabolites. Well-dispersed suspension cultures consist of thin-walled undifferentiated cells, but these are never uniform in size and shape. Cells with more differentiated structure, possessing, for example, thicker walls and even tracheid-like elements, usually only occur in large cell aggregates.

Many different methods of suspension culture have been developed. They fall into two main types: batch cultures in which cells are nurtured in a fixed volume of medium until growth ceases, and continuous cultures in which cell growth is maintained by continuous replenishment of sterile nutrient media. All techniques utilise some method of agitating the culture medium to ensure necessary cell dispersion and an adequate gas exchange.

Batch cultures. Batch cultures are initiated by inoculating cells into a fixed volume of nutrient medium. As growth proceeds, the amount of cell material increases until nutrients in the medium are depleted or there is the accumulation of an inhibitory metabolite. Batch cultures have a number of disadvantages that restrict their suitability for extended studies of growth and metabolism, or for the industrial production of plant cells, but they are nevertheless widely used for many laboratory investigations. Small cultures are frequently agitated on orbital shakers onto which are fixed suitable containers, which range in volume from 100 ml (Erlenmeyer conical flasks) to 1000 ml (spherical flasks); the quantity of medium being approximately the same as the flask volume. The shakers are usually operated at speeds from 30-180 rpm with an orbital motion of about 3 cm. Alternatively, stirred systems can be used.

Continuous cultures. Using batch cultures, it is difficult to obtain a steady rate of production of new cells having constant size and composition. Attempts to do so necessitate frequent sub-culturing, at intervals equivalent to the doubling time of the cell population. Satisfactorily balanced growth can only be produced in continuous culture, a method, which is especially important when plant cells are to be used for the large-scale production of a primary or secondary metabolite. Continuous culture techniques require fairly complicated apparatus. Agitation of larger cultures in bio-reactors is usually achieved by stirring with a turbine and/or by passing sterile air (or a controlled gaseous mixture) into the culture from below and releasing it through plugged vents. Mechanically stirred reactors damage plant cells by shearing. This is minimised in air-lift reactors. Different bioreactor designs are illustrated in Fig. 1.9.

The use of suspension cultures in plant propagation. The growth of plant cells is more rapid in suspension than in callus culture and is also more readily controlled because the culture medium can be easily amended or changed. Organs can be induced to develop in cell suspensions: root and shoot initiation usually commences in cell aggregates. Somatic embryos may arise from single cells. Cells from suspensions can also be plated onto solid media where single cells and/or cell aggregates grow into callus colonies from which plants can often be regenerated. For these reasons suspension cultures

might be expected to provide a means of very rapid plant multiplication. There are two methods:

- plants may be obtained from somatic embryos formed in suspensions. Once embryos have been produced, they are normally grown into plantlets on solid media, although other methods are potentially available (Chapter 2);
- cells from suspensions are plated onto solid media where single cells and/or cell aggregates grow into callus colonies from which plants can often be regenerated.

In practice neither of these techniques has been sufficiently reliable for use in plant propagation.

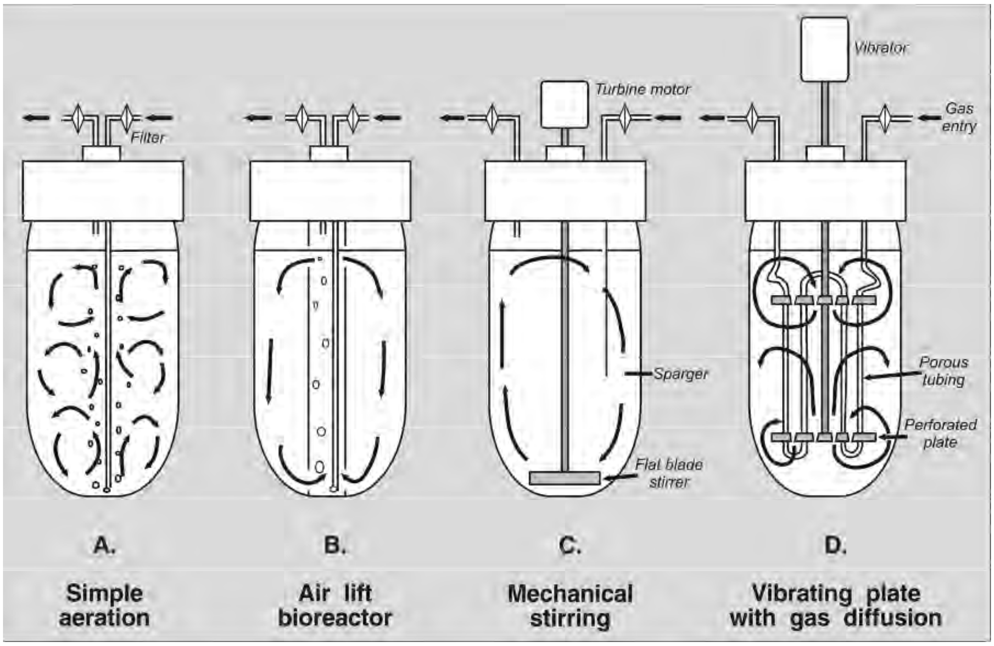

Fig.1.9 Four types of bioreactors used for plant cell culture.

Immobilised cell cultures. Plant cells can be captured and immobilised by being cultured in a gel which is afterwards solidified (see Chapter 4). This technique has only limited application to plant micropropagation, but is now employed quite widely when plant cells are grown for the production of their secondary products or for the bio-transformation of chemical compounds (Lindsey and Yeoman, 1983).

3.3. CULTURES OF SINGLE CELL ORIGIN

3.3.1. Single cell clones

Cultures can be initiated from single plant cells, but only when special techniques are employed. Frequently these comprise passing suspension-cultured cells through a filter which removes coarse cell aggregates and allows only single cells and very small cell clusters to pass through. Small groups of cells are then assumed to have originated from single cells. The suspension obtained is usually plated onto (or incorporated into) a solidified medium in Petri dishes at a sufficient density to permit cell growth (see below), but with the cells sufficiently dispersed so that, when growth commences, individual callus colonies can be recognised under a binocular microscope and transferred separately to fresh medium. Cell lines originating from single cells in this way are sometimes called single cell clones or cell strains. The derivation of single cell clones was reviewed by Street (1977c).

Each cell clone has a minimum effective initial cell density (or minimum inoculation density) below which it cannot be cultured. The minimum density varies according to the medium and growth regulators in which the cells are placed; it is frequently about 10–15 cells/ml on standard media. Widely dispersed cells or protoplasts will not grow because they lose essential growth factors into the surrounding medium. The minimum inoculation density can therefore be lowered by adding to a standard medium either a filtered extract of a medium in which a culture has

been previously grown (the medium is then said to be conditioned), or special organic additives (when it is said to be supplemented).

Cells or protoplasts (see below) plated at a density which is insufficient for spontaneous cell division may also be nurtured into initial growth by being 'nursed' by tissue growing nearby. One way of doing this is to place an inoculum onto a filter paper disc (a raft) or some other inert porous material, which is then put in contact with an established callus culture of a similar species of plant, the cells of which are called nurse cells, and the tissue a feeder layer. An alternative technique is to divide a Petri dish into compartments (Fig. 1.10.). Nurse tissues cultured in some segments assist the growth of cells or protoplasts plated in the other areas.

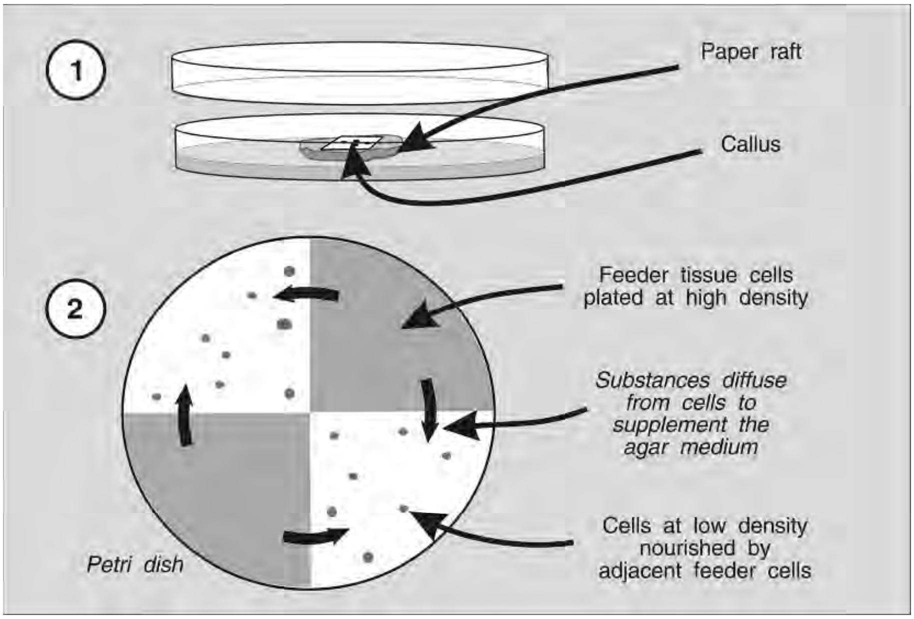

Fig. 1.10 Two methods of assisting the growth of cells plated at low density.

Another method of producing cell colonies which are very likely to have had a single cell origin, has been described by Bellincampi *et al.* (1985). A filtered cell suspension with a high proportion of single cells, is cultured at high density in a medium which contains only 0.2% agar. At this concentration the agar does not solidify the medium, but keeps apart the cell colonies growing from individual cells, preventing them from aggregating. When clusters of approximately 10-15 cells have been formed, they can be plated at a dilution of 50 (20% plating efficiency) to 200 plating units/ml (60% plating efficiency) on a medium gelled with 1% agar where they grow as separate callus colonies. Plating efficiency is the percentage of plating units (cell aggregates in this case) which give rise to callus colonies

The establishment of single cell clones is one way to separate genetically different cell lines from a mixed cell population. By artificially increasing the genetic variation between cells in a culture, and then applying a specific selection pressure, resistant cell lines have been obtained (e.g. those resistant to certain drugs, herbicides or high levels of salt), and in some instances plants with similar resistances have then been regenerated from the resulting cells or callus (Dix, 1990).

3.3.2. Separated cells

Single cells can be separated directly from intact plants. They are often more easily isolated and less liable to damage than protoplasts, because the cell wall remains intact. Consequently, single cells can be used in robust operations, such as direct physiological studies. It has been said that, for this purpose, they are more representative of differentiated tissues than cells derived from tissue cultures (Miksch and Beiderbeck, 1976); but the disruption caused by separation may induce atypical responses.

Mechanical separation. In some plant species, disrupting the tissue mechanically can separate intact cells of certain organs. Viable mesophyll cells, for example, can be obtained easily from *Asparagus cladodes* (Colman *et al.,* 1979) and from leaves of *Macleaya cordata* (Kohlenbach; 1966, 1967). These cells can be grown either in suspension or solid culture and induced into morphogenesis, including somatic embryo formation (Kohlenbach, 1977). Schwenk (1980, 1981) simply placed pieces of the young cotyledons of sweet potato in water inside an abrasive tube in which a vortex was created. After removing debris, a cell suspension could be obtained from which cells grew and formed callus when plated on nutrient agar.

However, the capacity to isolate separated cells directly from higher plants appears to be limited (Jullien and Rossini, 1977). The type of tissue used seems to be important both to permit cell separation and to obtain subsequent growth. Cells separated from the leaves, instead of from the cotyledons, of sweet potato (above) had no capacity for growth, and it was not possible to even separate cells by mechanical means from several other plants.

Enzymatic separation. Cell separation can be assisted by treating plant tissue with enzyme preparations such as crude pectinase or polygalacturonase, which loosen the attachment between individual cells in a tissue. Zaitlin first used this technique in 1959 to separate viable cells from tobacco leaves. Methods of isolation have been described by Takebe *et al.* (1968); Servaites and Ogren (1977) and Dow and Callow (1979). Cells isolated in this way can be suspended in culture medium and remain metabolically active.

Separated cells from leaf tissue of tobacco pre-infected with Tobacco Mosaic Virus have been used to study the formation of viral RNA's in the infected cells, and for studies on the interaction between leaf tissue cells and elicitor chemicals produced by fungal pathogens (Dow and Callow, 1979). Button and Botha (1975) produced a suspension of single cells of *Citrus* by macerating callus with 2-3% Macerase enzyme: the degree of dispersion of cells from suspension cultures can also be improved by enzyme addition (Street, 1977c).

3.3.3. Protoplasts

A protoplast is the living part of a plant cell, consisting of the cytoplasm and nucleus with the cell wall removed. Protoplasts can be isolated from whole plant organs or tissue cultures. If they are then placed in a suitable nutrient medium, they can be induced to re-form a cell wall and divide. A small cluster of cells eventually arises from each cell and, providing the protoplasts were originally plated at a relatively low density, can be recognised as one of many discrete 'callus colonies'. Plants can often be regenerated from such callus. Protoplast culture therefore provides one route whereby plants can be multiplied, but it is not yet used for routine micropropagation work, although the number of species in which plant regeneration has been achieved is steadily increasing.

At present isolated protoplasts are used chiefly in research into plant virus infections, and for modifying the genetic information of the cell by inserting selected DNA fragments. Protoplasts may also be fused together to create plant cell hybrids. Genetically modified cells will be only of general practical value if whole plants having the new genetic constitution can be regenerated from them. The ability to recover plants from protoplast cultures is therefore of vital importance to the success of such genetic engineering projects in plant science.

Methods of protoplast preparation. There are several different methods by which protoplasts may be isolated:

- by mechanically cutting or breaking open the cell wall;
- by digesting away the cell wall with enzymes;
- by a combination of mechanical and enzymatic separation.

For successful isolation it has been found essential to cause the protoplast to contract away from the cell wall, to which, when the cell is turgid, it is tightly adpressed. Contraction is achieved by plasmolysing cells with solutions of salts such as potassium chloride and magnesium sulphate, or with sugars or sugar alcohols (particularly mannitol) (see Chapter 4). These osmotica must be of sufficient concentration to cause shrinkage of the protoplasm, but of insufficient strength to cause cellular damage.

In the past, protoplasts have been mechanically isolated from pieces of sectioned plant material, but only very small numbers were obtained intact and undamaged. This method has therefore been almost completely replaced by enzymatic isolation techniques. Commercially available preparations used for protoplast isolation are often mixtures of enzymes from a fungal or bacterial source, and have pectinase, cellulase and/or hemicellulase activity: they derive part of their effectiveness from being of mixed composition (Evans and Cocking, 1977).

Protoplasts are usually isolated using a combination of several different commercial products. Plasmolysis helps to protect the protoplast when the cell wall is ruptured during mechanical separation and also appears to make the cell more resistant to the toxic effects of the enzymes used for cell wall digestion. It also severs the plasmodesmata linking adjacent cells and so prevents the amalgamation of protoplasms when the cell walls are digested away.

Tissue from an entire plant to be used for protoplast separation, is first surface sterilised. Some further preparation to allow the penetration of osmotic solutions and the cell wall degrading enzymes, is often advantageous. For instance, when protoplasts are to be separated from leaf mesophyll, the epidermis of the leaf is first peeled away, or the leaf is cut in strips and the tissue segments are then plasmolysed. The next step is to incubate the tissue with pectinase and cellulase enzymes for up to 18 hours in the same osmoticum, during which time the cell walls are degraded. Agitation of the incubated medium after this interval causes protoplasts to be released. They are washed and separated in solutions of suitable osmotic potential before being transferred to a culture medium.

Less severe and prolonged enzymatic cell digestion is required if plant tissue is first treated to mild mechanical homogenisation before cellulase treatment. Another technique calls for the sequential use of enzymes; firstly pectinase to separate the cells, and then, when separation is complete, cellulase to digest the cell walls. The yield of viable protoplasts can sometimes be increased by pre-treatment of the chosen tissue with growth substances before separation is attempted (Kirby and Cheng, 1979). Protoplasts are also commonly isolated by enzymatic treatment of organs or tissues that have been cultured *in vitro*. Cells from suspension cultures, which have been subcultured frequently, and are dividing rapidly, are one suitable source.

The successful isolation of viable protoplasts capable of cell division and growth, can depend on the manner in which the mother plant was grown. For example, Durand (1979) found that consistently successful protoplast isolation from haploid *Nicotiana sylvestris* plants depended on having reproducible batches of young plants *in vitro*. The composition of the medium on which these plants were cultured had a striking effect on protoplast yield and on their ability to divide. A low salt medium devoid of vitamins was particularly disadvantageous. The light intensity under which the plants were grown was also critical.

Protoplast culture. Isolated plant protoplasts are very fragile and particularly liable to either physical or chemical damage. Thus if they are suspended in a liquid medium, it must not be agitated, and the high osmotic potential of the medium in which isolation was carried out must be temporarily maintained. As growth depends on adequate aeration, protoplasts are usually cultured in very shallow containers of liquid or solid media; fairly high plating densities (5×10^4 to 10^5 protoplasts/ml) may be necessary, possibly because endogenous chemicals are liable to leak away from such unprotected cells. To promote growth, it may also be beneficial to add to the medium supplementary chemicals and growth factors not normally required for the culture of intact cells.

The capability of plant protoplasts to divide appears to be closely related to their ability to form a cell wall (Meyer and Abel, 1975a,b). The type of wall that is produced initially can be controlled to some extent by the nature of the culture medium. A non-rigid wall can be produced on tobacco mesophyll protoplasts, for example, by culture in a medium containing a relatively high concentration of salts; but although such cells will divide 2–3 times, further cell division does not occur unless a rigid wall is induced to be formed by a change in the culture medium (Meyer, 1974). Under favourable circumstances formation of a cell wall seems to occur as soon as protoplasts are removed from hydrolysing enzyme preparations, and the first signs of cellulose deposition can be detected after only about 16 hours in culture medium. Once wall formation is initiated, the concentration of osmoticum is reduced to favour cell growth. This is readily accomplished in a liquid medium, but where protoplasts have been plated onto a solidified medium it will be necessary to transfer the cells on blocks of agar, to another substrate.

When it has formed a cell wall, the regenerated plant cell generally increases in size and may divide in 3–5 days. If further cell divisions occur, each protoplast gives rise to a small group of intact cells and then a small callus colony. Green chloroplasts in cells derived from leaf mesophyll protoplasts, lose their integrity and disappear as callus formation proceeds. Protoplasts may originate from cells of the intact plant, which are not all of the same genetic composition. If such cells are grown in liquid medium, they may stick together and form common cell walls. Colonies of mixed callus will result which

could give rise to genetically different plants (see Chapter 3) or plant chimeras (D'Amato, 1978).

To avoid cell aggregation, protoplasts should be freely dispersed and cultured at as low a density as possible. This may mean that, as in the culture of intact cells at low density (see above), nurse tissue, or a conditioned or specially supplemented medium, must be employed. A method of the latter kind was devised by Raveh *et al.* (1973). A fabric support has been used to suspend protoplasts in a liquid medium so that media changes can be made readily (Kirby and Cheng, 1979).

For further information, readers should consult one or other of the following references:

- Bajaj (1977), Evans and Cocking (1977) and Evans and Bravo (1983), who provide good basic reviews of the subject
- Gamborg *et al.* (1981), describe methods and protocols for protoplast isolation, culture (and fusion)
- Constabel (1982) and Fowke (1982a), chapters describing methods and equipment for protoplast isolation and culture

An entire plant was first regenerated from callus originated from an isolated protoplast in 1971 (Takebe *et al.,* 1971). Since then plants have been produced from the protoplasts of a wide range of species, using indirect shoot morphogenesis or indirect embryogenesis (Davey and Power, 1988). The direct formation of somatic embryos (see below) from cultured protoplasts is also possible (Zapata and Sink, 1980).

Protoplast fusion. Although fusion of plant protoplasts was observed many years ago, it has become especially significant since methods have been developed for protoplast isolation and subsequent regeneration into intact plants. Isolated protoplasts do not normally fuse together because they carry a superficial negative charge causing them to repel one another. Various techniques have been discovered to induce fusion to take place. Two of the most successful techniques are the addition of polyethylene glycol (PEG) in the presence of a high concentration of calcium ions and a pH between 8-10, and the application of short pulses of direct electrical current (electro-fusion). By mixing protoplasts from plants of two different species or genera, fusions may be accomplished:

- (a) between protoplasts of the same plant where fusion of the nuclei of two cells would give rise to a homokaryon (synkaryon);
- (b) between protoplasts of the same plant species (intravarietal or intraspecific fusion);
- (c) between protoplasts of different plant species or genera (interspecific or intergeneric fusion).

Fusions of types (b) and (c) above can result in the formation of genetic hybrids (heterokaryocytes), which formally could only be obtained rarely through sexual crossings. By separating the fused hybrid cells from the mixed protoplast population before culture, or by devising a method whereby the cells arising from fused cells may be recognised once they have commenced growth, it has been possible to regenerate new somatic hybrid (as opposed to sexually hybrid) plants. Some novel interspecific and intergeneric hybrid plants have been obtained by this means. A fusion of the cytoplasm of one kind of plant with the nucleus of another is also possible. Such cybrid plants can be useful in plant breeding programmes for the transfer of cytoplasmic genes.

The following references give further details about this research topic and its implications for crop improvement:

- Schieder and Vasil (1980). A well-referenced review which lists somatic hybrid cell lines or plants obtained by protoplast fusion.
- Ferenczy and Farkas (1980) is a book on protoplast research in fungi, yeasts and plants. Several papers describe the results of fusions between protoplasts of different plant species or genera.
- Dodds and Roberts (1982), a short chapter describing methods and techniques.
- Keller *et al.* (1982), a useful review of the production and characterisation of somatic hybrids and the practical applications of protoplast fusion technology.
- Kao (1982) and Fowke (1982a,b) describe protocols for protoplast fusions in great detail.
- Mantell *et al.* (1985). An introduction to plant genetic engineering of various kinds.
- Glimelius (1988). Uses of protoplast fusion for plant breeding objectives.
- Davey and Power (1988). Progress in protoplast culture, fusion and plant regeneration

4. CYTODIFFERENTIATION

In an intact plant there are many kinds of cells all having different forms and functions. Meristematic cells, and soft thin-walled parenchymatous tissue, are said to be undifferentiated, while specialised cells are

said to be differentiated. The cells of callus and suspension cultures are mainly undifferentiated, and it is not yet possible to induce them to become of just one differentiated type. This is partly because culture systems are usually designed to promote cell growth: differentiation frequently occurs as cells cease to divide actively and become quiescent. Furthermore, the formation of differentiated cells appears to be correlated with organ development, therefore the prior expression of genes governing organogenesis may often be required. The *in vitro* environment can also be very different to that in the whole plant where each cell is governed by the restraint and influence of other surrounding cells. In suspension cultures, for example, cells are largely deprived of directional signals, influences from neighbouring differentiated tissues, and correlative messages that may normally pass between adjacent cells by way of interconnecting strands of protoplasm (plasmodesmata).

The differentiated state is also difficult to preserve when cells are isolated from a plant. Askani and Beiderbeck (1988) tried to keep mesophyll cells in a differentiated state. The character of palisade parenchyma cells with regard to size, cell form, colour and size, and distribution of chloroplasts could be preserved for 168h, but after this the chloroplasts became light green, their distribution was no longer homogeneous and some of the cells began to divide. Differentiated cells are most effectively produced *in vitro* within organs such as shoots and roots; even here there may not be the full range of cell types found in intact plants *in vivo*.

4.1. DIFFERENTIATED CELLS IN CALLUS AND CELL CULTURES

Three types of differentiated cells are commonly found in callus and cell cultures; these are vessels and tracheids (the cells from which the water-conducting vascular xylem is constructed), and cells containing chloroplasts (organelles carrying the green photosynthetic pigment, chlorophyll). Phloem sieve tubes may be present but are difficult to distinguish from undifferentiated cells.

4.1.1. Tracheid formation

Callus cultures are more likely to contain tracheids than any other kind of differentiated cell. The proportion formed depends on the species from which the culture originated and especially upon the kind of sugar and growth regulators added to the medium. This is discussed further in Chapter 10. Tracheid formation may represent or be associated with an early stage in the development of shoot meristems. Nodules containing xylem elements in callus of *Pelargonium* have, for example, been observed to develop into shoots when moved to an auxin-free medium (Chen and Galston, 1967; Cassells, 1979).

The rapid cell division initiated when tissue is transferred to a nutrient medium usually occurs in meristems formed around the periphery of the explant. Cell differentiation does not take place in callus cultures during this phase but begins when peripheral meristematic activity is replaced or supplemented by the formation of centres of cell division deeper in the tissue. These internal centres generally take the form of meristematic nodules that may produce further expanded and undifferentiated cells (so contributing to callus growth) or cells that differentiate into xylem or phloem elements. Nodules can form primitive vascular bundles, with the xylem occurring centrally and the phloem peripherally, separated from the xylem by a meristematic region.

4.1.2. Chloroplast differentiation

The formation and maintenance of green chloroplasts in cultured plant cells represents another form of cellular differentiation which is easy to monitor, and which has been studied fairly extensively. When chloroplast-containing cells from an intact plant are transferred to a nutrient medium they begin to dedifferentiate. This process continues in the event of cell division and results in a loss of structure of the membranes containing chlorophyll (thylakoids) and the stacks (grana) into which they are arranged, and the accumulation of lipid-containing globules. The chloroplasts eventually change shape and degenerate.

Callus cells frequently do not contain chloroplasts but only plastids containing starch grains in which a slightly-developed lamellar system may be apparent. All the same, many calluses have been discovered that do turn green on continued exposure to light and are composed of a majority of chloroplast-containing cells. Chloroplast formation can also be connected with the capacity of callus to undergo morphogenesis. Green spots sometimes appear on some calluses and it is from these areas that new shoots arise. By subculturing areas with green spots, a highly morphogenic tissue can sometimes be obtained. The formation of chloroplasts and their continued integrity is also favoured by cell aggregation. When

green callus tissue is used to initiate suspension cultures, the number of chloroplasts and their degree of differentiation are reduced. Nevertheless, there can be some increase in chlorophyll content during the stationary phase of batch cultures.

The level of chlorophyll so far obtained in tissue cultures is well below that found in mesophyll cells of whole plants of the same species, and the rate of chlorophyll formation on exposure of cultured cells to the light is extremely slow compared to the response of etiolated organised tissues. The greening of cultures also tends to be unpredictable and even within individual cells, a range in the degree of chloroplast development is often found. In the carbon dioxide concentrations found in culture vessels, green callus tissue is normally photomixotrophic (i.e. the chloroplasts are able to fix part of the carbon that the cells require) and growth is still partly dependent on the incorporation of sucrose into the medium (Vasil and Hildebrandt, 1966). However, green photoautotrophic callus cultures have been obtained from several different kinds of plants. When grown at high carbon dioxide concentrations (1–5%), without a carbon source in the medium, they are capable of increasing in dry weight by photosynthetic carbon assimilation alone (see Street, 1977a).

Photoautotrophic cell suspensions have also been obtained. They too normally require high carbon dioxide levels, but cell lines of some species have been isolated capable of growing in ambient CO_2 concentration (Xu *et al.,* 1988). Why cultured cells do not freely develop fully functional chloroplasts is not fully known. Some hypotheses have been summarised by Dalton (1980). The cytology of chloroplast formation is described in Yeoman and Street (1977). Photoautotrophic growth of shoots is described in Chapter 2.

5. MORPHOGENESIS

5.1. NATURE AND INDUCTION

New organs such as shoots and roots can be induced to form on cultured plant tissues. Such freshly originated organs are said to be adventive or adventitious. The creation of new form and organisation, where previously it was lacking, is termed morphogenesis or organogenesis. Tissues or organs that have the capacity for morphogenesis/organogenesis are said to be morphogenic (morphogenetic) or organogenic (organogenetic). So far it has been possible to obtain the *de novo* (adventitious) formation of:

- shoots (caulogenesis) and roots (rhizogenesis) separately. The formation of leaves adventitiously *in vitro* usually denotes the presence of a shoot meristem. Sometimes leaves appear without apparent shoot formation: opinions are divided on whether such leaves can have arisen *de novo*, or whether a shoot meristem must have been present first of all and subsequently failed to develop.
- embryos that are structurally similar to the embryos found in true seeds. Such embryos often develop a region equivalent to the suspensor of zygotic embryos and, unlike shoot or root buds, come to have both a shoot and a root pole. To distinguish them from zygotic or seed embryos, embryos produced from cells or tissues of the plant body are called *somatic embryos* (or *embryoids*) and the process leading to their inception is termed *embryogenesis*. The word '*embryoid*' has been especially used when it has been unclear whether the embryo-like structures seen in cultures were truly the somatic equivalent of zygotic embryos. Somatic embryogenesis is now such a widely observed and documented event that *somatic embryo* has become the preferred term.
- flowers, flower initials or perianth parts. The formation of flowers or floral parts is rare, occurring only under special circumstances and is not relevant to plant propagation.

6. HAPLOID PLANTS

6.1. ANTHER AND POLLEN CULTURE

In 1953 Tulecke discovered that haploid tissue (i.e. tissue composed of cells having half the chromosome number that is characteristic of a species), could be produced by the culture of *Ginkgo* pollen. Little notice was taken of his work until Guha and Maheshwari (1964, 1967) managed to regenerate haploid plants from pollen of *Datura innoxia* by culturing intact anthers. Since then a great deal of research has been devoted to the subject.

The basis of pollen and anther culture is that on an appropriate medium the pollen microspores of some

plant species can be induced to give rise to vegetative cells, instead of pollen grains. This change from a normal sexual gametophytic pattern of development into a vegetative (sporophytic) pattern, appears to be initiated in an early phase of the cell cycle when transcription of genes concerned with gametophytic development is blocked and genes concerned with sporophytic development are activated (Sunderland and Dunwell, 1977). The result is that in place of pollen with the capacity to produce gametes and a pollen tube, microspores are produced capable of forming haploid pro-embryos (somatic embryos formed directly from the microspores), or callus tissue. The formation of plants from pollen microspores in this way is sometimes called androgenesis. Haploid plants are more readily regenerated by culturing microspores within anthers than by culturing isolated pollen. The presence of the anther wall provides a stimulus to sporophytic development. The nature of the stimulus is not known but it may be nutritional and/or hormonal. Embryogenesis has only been induced from isolated pollen of a very small number of plants.

The number of plants species from which anther culture has resulted in haploid plants is relatively few. It comprised about 70 species in 29 genera up to 1975 (Sunderland and Dunwell, 1977) and 121 species or hybrids in 20 families by 1981-1982 (Maheshwari *et al.,* 1982) and by now, very many more. The early stages of embryogenesis or callus formation without plant regeneration have been obtained in several other kinds of plants. Fifty- eight per cent of the reports of embryogenesis or plant regeneration in Maheshwari *et al.* (1982) was attributable to species within the family Solanaceae. Species in which haploid plants can be regenerated reliably and at high frequency remain a comparatively small part of the total. They again mainly comprise Solanaceous species such as *Datura*, *Nicotiana*, *Hyoscyamus*, *Solanum* and some brassicas.

For further information on pollen and anther culture, which is outside the scope of the present book, the reader should consult the following books or review articles: Dunwell (1985); Foroughi-Wehr and Wenzel (1989); Giles and Prakash (1987); Heberle-Bors (1985); Hu and Yang (1986); Jain *et al.* (1996); Keller and Stringham (1978); Maheshwari *et al.* (1980, 1982); Morrison and Evans (1988); Nitsch (1977, 1981; 1983); Palmer *et al.* (2005); Raghavan (1990); Reinert and Bajaj (1977); Sangwan and Sangwan-Norreel (1990); Vasil (1980c).

6.2. GYNOGENESIS

Another theoretical source of haploid plants in angiosperms is the female egg nucleus or ovum; this is contained within the nucellus of an ovule in a specialised cell (the megaspore or embryo sac). The ovum cannot be separated readily from other associated nuclei in the megaspore and so haploid plants can normally be produced from it, only by stimulating the development of unfertilised ovules into seedlings. In some species [e.g. *Gerbera jamesonii* (Sitbon, 1981; Meynet and Sibi, 1984); maize (Truong-Andre and Demarly, 1984); sugar beet (Hosemans and Bossoutrot, 1983); onion (Keller, 1990)], some haploid plants can be obtained by culturing unpollinated ovules, ovaries or flower buds. In some other plants (Pavlova, 1986), larger numbers of haploids are obtained if ovaries are pollinated by a distantly-related species (or genus) or with pollen which has been irradiated with X- or γ-rays. Successful pollination results in stimulation of endosperm growth by fusion of one of the generative nuclei of the pollen tube with the central fusion nucleus of the megaspore, but fusion of the other generative nucleus with the egg cell does not occur and the egg cell is induced to grow into a seedling without being fertilised (gynogenesis). An alternative technique, which has resulted in haploid *Petunia* seedlings (Raquin, 1986) is to treat ovaries with γ-rays and then pollinate them with normal pollen. Gynogenesis has so far been employed much less frequently than androgenesis for the production of haploids. A review of progress in this area has been provided by Yang and Zhou (1990).

Haploid cells and haploid plants produced by androgenesis or gynogenesis have many uses in plant breeding and genetics (Vasil and Nitsch, 1975). Most recent research on anther culture has concentrated on trying to improve the efficiency of plantlet regeneration in economically important species. Haploid plants of cereals are particularly valuable in breeding programmes, but in the Gramineae, the frequency and reliability of recovery through anther culture is still too low for routine use.

REFERENCES

ANGRISH R. & NANDA K.K. 1982a Seasonal culture of dormant reproductive buds of *Salix tetrasperma*: analysis of the flowering process. Plant Cell Tissue Organ Cult. 1, 181-193.

ANGRISH R. & NANDA K.K. 1982b Dormancy and flowering process in reproductive buds of *Salix babylonica* cultured *in vitro*. Z. Pflanzenphysiol. 106, 263-26.

ANON 1983 *Cell and Tissue Culture Techniques for Cereal Crop Improvement*. Science Press, Beijing, China. Gordon and Breach, Science Publishers Inc., New York.

ASKANI A. & BEIDERBECK R. 1988 Preservation of the differentiated state of mesophyll cells *in vitro*. Ann. Bot. 61, 393-394.

BAJAJ Y.P.S. 1977 Protoplast isolation, culture and somatic hybridization. pp. 467-496 in Reinert and Bajaj (eds.) 1977 (q.v.).

BALLADE P. 1971 Étude expérimentale de l'organogenèse axillaire chez *Nasturtium officinale* R.Br. Comportement de noeuds isolés cultivés *in vitro*. Compt. Rend. Acad. Sci. Paris 273D, 2079-2082.

BARZ W., REINHARD E. & ZENK M.H. (eds.) 1977 *Plant Tissue Culture and its Biotechnological Application.* Springer-Verlag, Berlin, Heidelberg, New York.

BELLINCAMPI D., BADURI N. & MORPURGO G. 1985 High plating efficiency with plant cell cultures. Plant Cell Rep. 4, 155-157.

BERGHOEF J. & BRUINSMA J. 1979a Flower development of *Begonia franconis* Liebm. II. Effects of nutrition and growth-regulating substances on the growth of flower buds *in vitro*. Z. Pflanzenphysiol. 93, 345-357.

BERGHOEF J. & BRUINSMA J. 1979b Flower development of *Begonia franconis* Liebm. III. Effects of growth regulating substances on organ initiation in flower buds *in vitro*. Z. Pflanzenphysiol. 93, 377-386.

BILDERBACK D.E. 1971 The effects of amino acids upon the development of excised floral buds of *Aquilegia*. Am. J. Bot. 58, 203-208.

BLAKE J. 1966 Flower apices cultured *in vitro*. Nature 211, 990-991.

BLAKE J. 1969 The effect of environmental and nutritional factors on the development of flower apices cultured *in vitro*. J. Exp. Bot. 20, 113-123.

BUTCHER D.N. & STREET H.E. 1964 Excised root culture. Bot. Rev. 30, 513-586.

BUTTON J. & BOTHA C.E.J. 1975 Enzymic maceration of *Citrus* callus and the regeneration of plants from single cells. J. Exp. Bot. 26, 723-729.

CAPONETTI J.D. & STEEVES I.M. 1963 Morphogenetic studies on excised leaves of *Osmunda cinnamomea* L. Morphological studies of leaf develpment in sterile nutrient culture. Can. J. Bot. 41, 545-556.

CAPONETTI J.D. 1972 Morphogenetic studies on excised leaves of *Osmunda cinnamomea*. Morphological and histological effects of sucrose in sterile nutrient culture. Bot. Gaz. 133, 421-435.

CASSELLS A.C. & DOYLE B.M. 2005 Pathogen and biological contamination management: the road ahead. pp. 35-50 in Loyola-Vargas V.M. & Vázquez-Flota F. (eds.). *Plant Cell Culture Protocols.* Humana Press, New York.

CASSELLS A.C. (ed.) 1997 *Pathogen and Microbial Contamination Management in Micropropagation.* Kluwer, Dordrecht.

CASSELLS A.C. 1979 The effect of 2,3,5-tri-iodobenzoic acid on caulogenesis in callus cultures of tomato and *Pelargonium*. Physiol. Plant. 46, 159-164.

CHEN H.-R. & GALSTON A.W. 1967 Growth and development of *Pelargonium* pith cells *in vitro*. II. Initiation of organised development. Physiol. Plant. 20, 533-539.

CLAPHAM D.H. 1977 Haploid induction in cereals. pp. 279-298 in Reinert & Bajaj (eds.) 1977 (q.v.).

COLLINS G.B. & GROSSER J.W. 1984 Culture of embryos. pp. 241-257 in Vasil I.K. (ed.) 1984 *Cell Culture and Somatic Cell Genetics of Plants* Vol. 1. *Laboratory Procedures and their Applications*. Academic Press, New York.

COLLINS G.B. 1978 Pollen and anther cultures -progress and applications. pp. 179-188 in Hughes *et al.* (eds.) 1978 (q.v.).

COLMAN B., MAWSON B.T. & ESPIE G.S. 1979 The rapid isolation of photosynthetically active mesophyll cells from *Asparagus* cladophylls. Can. J. Bot. 57, 1505-1510.

CONSTABEL F. 1982 Isolation and culture of plant protoplasts. pp. 38-48 in Wetter and Constabel (eds.) 1982 (q.v.).

DALTON C.C. 1980 Photoautotrophy of spinach cells in continuous culture: Photosynthetic development and sustained photoautotrophic growth. J. Exp. Bot. 31, 791-804.

D'AMATO F. 1978 Chromosome number variation in cultured cells and regenerated plants. pp. 287-295 in Thorpe T.A. (ed.) 1978 *Frontiers of Plant Tissue Culture*. Int. Assoc. Plant Tiss. Culture, University Calgary Printing Services, Calgary, Canada.

DANCKWARDT-LILLIESTROM C. 1957 Kinetin induced shoot formation from isolated roots of *Isatis tinctoria*. Physiol. Plant. 10, 794-797.

DAVEY M.R. & POWER J.B. 1988 Aspects of protoplast culture and plant regeneration. Plant Cell Tissue Organ Cult. 12, 115-125.

DE JONG A.W. & BRUINSMA J. 1974 Pistil development in *Cleome* flowers III. Effects of growth-regulating substances on flower buds of *Cleome iberidella* Welv. ex Oliv. grown *in vitro*. Z. Pflanzenphysiol. 73, 142-151.

DE NETTANCOURT D. & DEVREUX M. 1977 Incompatibility and *in vitro* cultures. pp. 426-441 in Reinert & Bajaj (eds.) 1977 (q.v.).

DEATON M., BUXTON J.W. & KEMP T.R. 1980 Senescence studies on *Nicotiana offinis* flowers cultured *in vitro*. HortScience 15, 433 (Abst. 460).

DIX P.J. (ed.) 1990 *Plant Cell Line Selection.* VCH, Weinheim.

DODDS J. H. & ROBERTS L. W. 1982 *Experiments in Plant Tissue Culture.* Cambridge University Press, Cambridge, London, New York.

DOW J.M. & CALLOW J.A. 1979 Leakage of electrolytes from isolated leaf mesophyll cells of tomato induced by glycopeptides from culture filtrates of *Fulvia fulva* (Cooke) Ciferri (syn. *Cladosporium fulvum*). Physiol. Plant Path. 15, 27-34.

DUNSTAN D.I., SHORT K.C. & THOMAS E. 1978 The anatomy of secondary morphogenesis in cultured scutellum tissues of *Sorghum bicolor*. Protoplasma 97, 251-260.

DUNWELL J.M. 1985 Anther and ovary culture. pp. 1-44 in Bright and Jones (eds) 1985 *Cereal Tissue and Cell Culture.* Martinus Nijhoff, Dortrecht.

DURAND J. 1979 High and reproducible plating efficiencies of protoplasts isolated from *in vitro* grown haploid *Nicotiana sylvestris* Spegaz. et Comes. Z. Pflanzenphysiol. 93, 283-296.

EVANS D.A. & BRAVO J.E. 1983 Plant protoplast isolation and culture. Int. Rev. Cytol. Suppl. 16, 33-53.

EVANS P.K. & COCKING E.C. 1977 Isolated plant protoplasts. pp. 103-135 in Street H.E. (ed.) 1977a (q.v.).

FERENCZY L. & FARKAS G.L. 1980 *Advances in plant protoplast research.* Proc. 5th Int. Protoplast Symposium, Szeged, Hungary 1979. Pergamon Press, Oxford.

FOROUGHI-WEHR B. & WENZEL G. 1989 Androgenetic haploid production. pp. 11-18 in I.A.P.T.C. Newsletter No. 58 July 1989.

FOWKE L.C. 1982a Isolation and culture of protoplasts from green algae. pp. 57-61 in Wetter and Constabel (eds.) 1982 (q.v.).

FOWKE L.C. 1982b Fusion of carrot and algal protoplasts. pp. 63-65 in Wetter and Constabel (eds.) 1982 (q.v.).

GALUN E., JUNG Y. & LANG A. 1962 Culture and sex modification of male cucumber buds *in vitro*. Nature 194, 596-598.

GAMBORG O.L., SHYLUK J.P. & SHAHIN E.A. 1981 Isolation, fusion and culture of plant protoplasts. pp. 115-153 in Thorpe T.A. (ed.) 1981 (q.v.).

GEILE W. & WAGNER E. 1980 Rapid development of cell suspension cultures from leaf sections of *Chenopodium rubrum* L. Plant Cell Environ. 3, 141-148.

GILES K.K. & PRAKASH J. 1987 Pollen: cytology and development. Int. Rev. Cytol. 107, 1-455.

GLIMELIUS K. 1988 Potentials of protoplast fusion in plant breeding programmes. Plant Cell Tissue Organ Cult. 12, 163-172.

GRANATEK C.H. & COCKERLINE A.W. 1979 Callus formation of cultured early differentiating barley embryos. B. Torrey Bot. Club 106, 85-96.

GREEN C.E. & PHILLIPS R.L. 1975 Plant regeneration from tissue cultures of maize. Crop Sci. 15, 417-427.

GUHA S. & MAHESHWARI S.C. 1964 *In vitro* production of embryos from anthers of *Datura*. Nature 204, 497.

GUHA S. & MAHESHWARI S.C. 1967 Development of embryoids from pollen grains of *Datura in vitro*. Phytomorph. 17, 454-461.

HEBERLE-BORS E. 1985 *In vitro* haploid formation from pollen: a critical review. Theor. Appl. Genet. 71, 361-374.

HICKS G.S. & SUSSEX I.M. 1970 Development *in vitro* of excised flower primordia of *Nicotiana tabacum*. Can. J. Bot. 48, 133-139.

HODGE M.H. 1986 Propagation of herbaceous perennials by root cuttings. Comb. Proc. Int. Plant Prop. Soc. 1985 35, 548-554.

HOSEMANS D. & BOSSOUTROT D. 1983 Induction of haploid plants from *in vitro* culture of unpollinated beet ovules (*Beta vulgaris* L.). Z. Pflanzenzucht. 91, 74-77.

HU C.-Y. & SUSSEX I. 1986 Suspensor and *in vitro* culture of immature soybean embryos. p. 41 in Somers *et al.* (eds.) 1986 (q.v.).

HU H. & YANG H. (eds.) 1986 *Haploids of Higher Plants* in vitro. Springer-Verlag, Heidelberg.

HUGHES K.W., HENKE R. & CONSTANTIN M. (eds.) 1978 *Propagation of higher plants through tissue culture.* Technical Inf. Center, U.S. Dept. Energy. Spingfield, Va. CONF-7804111.

HVOSLEF-EIDE A.K. & PREIL W. (eds.) 2005 *Liquid culture systems for* in vitro *plant propagation*. Springer, Dordrecht.

IWAI S., KISHI C., NAKATA K. & KUBO S. 1985 Production of a hybrid *Nicotiana repanda* Willd. × *N. tabacum* L. by ovule culture. Plant Science 41, 175-178.

JAIN S.M., SOKORY S.K. & VEILLEUX R.E. 1996 Haploid Production in Higher Plants – Vols. 1-4. Springer, Berlin.

ROSSINI L. 1977 Isolation of mesophyll cells from higher plant leaves: advantages and potentialities of a mechanical method. Ann. Amel. Plantes 27, 87-104.

KANTA K., RANGASWAMY N.S. & MAHESHWARI P. 1962 Test tube fertilisation in a flowering plant. Nature 194, 1214-1217.

KAO K.N. & MICHAYLUK M.R. 1975 Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126, 105-110.

KAO K.N. 1982 Plant protoplast fusion and isolation of heterokaryocytes. pp. 49-56 in Wetter and Constabel (eds.) 1982 (q.v.).

KATO M. & TOKUMASU S. 1983 Characteristics of F1 hybrids produced by ovule-culture in ornamental *Pelargonium*. Acta Hortic. 131, 247-252.

KELLER J. 1990 Haploids from unpollinated ovaries of *Allium cepa* - single plant screening, haploid determination, and long term storage.

KELLER W. A. & STRINGAM G. R. 1978 Production and utilisation of microspore-derived plants. pp. 113-122 in Thorpe T.A. (ed.) 1978 (q.v)

KELLER W.A., SETTERFIELD G., DOUGLAS G., GLEDDIE S. & NAKAMURA C. 1982 Production, characterization and utilization of somatic hybrids of higher plants. pp. 81-114 in Tomes *et al.* (eds.) 1982 (q.v.).

KERBAUY G.B. 1984a Regeneration of protocorm-like bodies through *in vitro* culture of root tips of *Catasetum* (Orchidaceae). Z. Pflanzenphysiol. 113, 287-291.

KERBAUY G.B. 1984b Plant regeneration of *Oncidium varicosum* (Orchidaceae) by means of root tip culture. Plant Cell Rep. 3, 27-29.

KIRBY E.G. & CHENG T-Y. 1979 Colony formation from protoplasts derived from Douglas-fir cotyledons. Plant Sci. Lett. 14, 145-154.

KOHLENBACH H.W. 1966 Die entwick lungspotengen explantierter und isolierter Dauerzellen. 1. Das Streckungt und Teilungswachstum isolierter Mesophyllzellen von *Macleaya cordata*. Z. Pflanzenphysiol. 55, 142-147.

KOHLENBACH H.W. 1967 The potentialities for development of explanted and isolated permanent cells. IV. The organogenic growth of *Macleaya cordata* calli. Z. Pflanzenphysiol. 57, 305-309.

KOHLENBACH H.W. 1977 Basic aspects of differentiation and plant regeneration from cell and tissue cultures. pp.355-366 in Barz, Reinhard and Zenk (eds.) 1977 (q.v.).

KRUSE P.F. & PATTERSON M.K. Jr. (eds.) *Tissue Culture Methods and Applications.* Academic Press, New York.

LARKIN P.J. & SCOWCROFT W.R. 1981 Somaclonal variation - a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60, 197-214.

LIM-HO C. L. 1982 Tissue culture of local orchid hybrids at the Singapore Botanic Gardens. pp. 295-300 in Rao A. N. (ed.) 1982 (q.v.).

LINDSEY K. & YEOMAN M.M. 1983 Novel experimental systems for studying the production of secondary metabolites by plant tissue culture. pp. 39-66 in Mantell and Smith (eds.) 1983 (q.v.).

MAHESHWARI P. & RANGA SWAMY N.S. (eds.) 1963 *Plant Tissue and Organ Culture*. Int. Soc. Plant Morphologists, Delhi, India.

MAHESHWARI S.C., RASHID A. & TYAGI A.K. 1980 Physiology of pollen haploid formation - the current status. pp. 393-398 in Sala *et al.* (eds.) 1980 (q.v.).

MAHESHWARI S.C., RASHID A. & TYAGI A.K. 1982 Haploids from pollen grains - retrospect and prospect. Am. J. Bot. 69, 865-879.

MANTELL S.H., & SMITH H. (eds.)1983 *Plant Biotechnology.* Soc. Exp. Biology Seminar, Series 18. Cambridge Univ. Press, Cambridge, London, New York.

MANTELL S.H., MATTHEWS J.A. & McKEE R.A. 1985 *Principles of Plant Biotechnology. An Introduction to Genetic*

Engineering in Plants. Blackwell Scientific Publications, Oxford. ISBN 0-632-01215-3.

MEYER M.M. Jr. 1974 Propagation of tall bearded Iris by *in vitro* techniques. HortScience 9, 271 (Abst. 22).

MEYER Y. & ABEL W.O. 1975a Importance of the cell wall for cell division and in the activity of the cytoplasm in cultured tobacco protoplasts. Planta 123, 33-40.

MEYER Y. & ABEL W.O. 1975b Budding and cleavage division of tobacco mesophyll protoplasts in relation to pseudo-wall and wall formation. Planta 125, 1-13.

MEYNET J. & SIBI M. 1984 Haploid plants from *in vitro* culture of unfertilized ovules in *Gerbera jamesonii*. Z. Pflanzenzucht. 93, 78-85.

MIKSCH U. & BEIDERBECK R. 1976 Mechanical isolation of single cells from leaves of higher plants. Biochem. Biophys. Pflanzen. 169, 191-196.

MONNIER M. 1990 Zygotic embryo culture. pp. 366-393 in Bhojwani S.S. (ed.) 1990 *Plant Tissue Culture: Applications and Limitations*. Elsevier, Amsterdam.

MORRISON R.A. & EVANS D.A. 1988 Haploid plants from tissue culture: new plant varieties in a shortened time frame. Bio/Technology 6, 684-690.

MUDGE K.W., BORGMAN C.A., NEAL J.C. & WELLER H.A. 1986 Present limitations and future prospects for commercial micropropagation of small fruits. Comb. Proc. Int. Plant Prop. Soc. 36, 538-543.

MURASHIGE T. & SKOOG F. 1962 A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15, 473-497.

NARAYANASWAMY S. 1977 Regeneration of plants from tissue cultures. pp. 179-206 in Reinert & Bajaj (eds.) 1977 (q.v.).

NIJKAMP H.J.J., VAN DER PLAS I.H.W. & VAN AARTRIJK J. (eds.) 1990 *Progress in Plant Cellular and Molecular Biology*. Proc. VIIth Int. Cong. on Plant Tissue and Cell Culture. Amsterdam, The Netherlands. 24-29 June 1990. Kluwer Academic Publishers, Dortrecht, Netherlands.

NITSCH C. 1977 Culture of isolated microspores. pp. 268-278 in Reinert & Bajaj (eds.) 1977 (q.v.).

NITSCH C. 1981 Production of isogenic lines: basic technical aspects of androgenesis. pp. 241-252 in Thorpe T.A. (ed.) 1981 (q.v.).

NITSCH C. 1983 Progress in anther and pollen culture techniques. pp. 1-10 in Anon. (ed.) 1983 (q.v.).

NITSCH J.P. 1951 Growth and development *in vitro* of excised ovaries. Am. J. Bot. 38, 566-577.

NITSCH J.P. 1963 The *in vitro* culture of flowers and fruits. pp. 198-214 in Maheshwari & Ranga Swamy (eds.) 1963 (q.v.).

NORSTOG K. 1979 Embryo culture as a tool in the study of comparative and developmental morphology. pp. 179-202 in Sharp *et al.* (eds.) 1979 (q.v.).

NORTON J.P. & BOLL W.G. 1954 Callus and shoot formation from tomato roots *in vitro*. Science 119, 220-221.

PALMER C.E., KELLER W.A. & KASHA K.J. (2005) *Haploids in Crop Improvement II.* Springer, Berlin.

PAVLOVA M.K. 1986 Experimental gynogenesis *in vitro*. p. 94 in Somers *et al.* (eds.) 1986 (q.v.).

PHILIP V.J. & NAINAR S.A.Z. 1986 Clonal propagation of *Vanilla planifolia* (Salisb.) Ames using tissue culture. J. Plant Physiol. 122, 211-215.

RAGGIO M., RAGGIO N. & TORREY J.G. 1957 The nodulation of isolated leguminous roots. Am. J. Bot. 44, 325-334.

RAGHAVAN V. 1967 pp. 413 in Wilt & Wessels (eds.) 1967 *Methods in Developmental Biology*. Thomas Y. Crowell, N. York.

RAGHAVAN V. 1977a Applied aspects of embryo culture. pp. 375-397 in Reinert and Bajaj (eds.) 1977 (q.v.).

RAGHAVAN V. 1977b Diets and culture media for plant embryos. pp. 361-413 in Recheigl M. Jr. (ed.) 1977 *CRC Handbook Series in Nutrition & Food* Vol. 4. CRC Press, Baton Rouge, Florida.

RAGHAVAN V. 1980 Embryo culture. pp. 209-240 in Vasil I.K. (ed.) 1980b (q.v.).

RAGHAVAN V. 1990 From microspore to embryoid: faces of the angiosperm pollen grain. pp. 213-221 in Nijkamp *et al.* (eds.) 1990 (q.v.).

RAMMING D.W. 1990 The use of embryo culture in fruit breeding. HortScience 25, 393-398.

RANGA SWAMY N.S. 1977 Applications of *in vitro* pollination and *in vitro* fertilization. pp. 412-425 in Reinert & Bajaj (eds.) 1977 (q.v.).

RAO A.N. (ed.) 1982 *Tissue Culture of Economically Important Plants*. Proc. Int. Symp., Singapore, 1981. COSTED and ANBS Publications.

RAQUIN C. 1986 Induction of haploid and "haploid plus" Petunias by combining gametophyte irradiation and *in vitro* ovary culture. p. 123 in Somers *et al.* (eds.) 1986 (q.v.).

RASTOGI R. & SAWHNEY V.K. 1988 *In vitro* culture of stamen primordia of the normal and a male sterile stamenless-2 mutant of tomato (*Lycopersicon esculentum* Mill.). J. Plant Physiol. 133, 349-352.

RAVEH D., HUBERMANN E. & GALUN E. 1973 *In vitro* culture of tobacco protoplasts: Use of feeder techniques to support division of cells plated at low densities. In Vitro 9, 216-222.

REINERT J. & BAJAJ Y. P. S. (eds.) 1977 *Plant Cell, Tissue and Organ Culture*. Springer-Verlag, Berlin.

REUTHER G. 1977 Adventitious organ formation and somatic embryogenesis in callus of *Asparagus* and *Iris* and its possible application. Acta Hortic. 78, 217-224.

RUDDAT M., KOKONTIS J. & SHOUP J.R. 1979 *In vitro* culture of isolated male and female flower buds from *Silene alba*. Plant Physiol. 63, (Suppl. 5) Abst. 759.

SAID A.G.E. & MURASHIGE T. 1979 Continuous cultures of tomato and citron roots *in vitro*. In Vitro 15, 593-602.

SALA F., PARISI B., CELLA R. & CIFERRI O (eds.) 1980 *Plant Cell Cultures: Results and Perspectives*. Elsevier/North Holland, Amsterdam, New York, ISBN 0-444-80204-5.

SANDERS M.E. & ZIEBUR N.K. 1958 Artificial culture of embryos. pp. 297-325 in Maheshwari P. (ed.) *Recent Advances in Embryology of Angiosperms.* Univ. of Delhi: Int. Soc. for Plant Morphology.

SANGWAN R.S. & SANGWAN-NORREEL B.S. 1990 Anther and pollen culture. In Bhojwani S.S. (ed.) 1990 *Plant Tissue Culture: Applications and Limitations.* Elsevier, Amsterdam.

SCHIEDER O. & VASIL I.K. 1980 Protoplast fusion and somatic hybridization. pp. 21-46 in Vasil I.K. (ed.) 1980b (q.v.).

SCHWENK F.W. 1981 Callus formation from mechanically isolated mesophyll cells of soybean and sweet potato. Plant Sci. Lett. 23, 147-151.

SCHWENK F.W.A 1980 Callus formation from mechanically isolated soybean cotyledonary cells. Plant Sci. Lett. 17, 437-442.

SEELIGER I. 1956 Uber die Kultur isolierter Wurzeln der Robinie (*Robinia pseudoacacia* L.). Flora 144, 47-83.

SERVAITES J.C. & OGREN W.L. 1977 Rapid isolation of mesophyll cells from leaves of soybean for photosynthetic studies. Plant Physiol. 59, 587-590.

SHARMA D.R., CHOWDHURY J.B., AHUJA U. & DHANKHAR B.S. 1980 Interspecific hybridization in genus

Solanum, a cross between *S. melongena* and *S. khasianum* through embryo culture. Z. Pflanzenzucht. 85, 248-253.

SHARP W.R., LARSEN P.O., PADDOCK E.F. & RAGHAVAN V. (eds.) 1979 *Plant Cell and Tissue Culture*. Ohio State Univ. Press, Columbus.

SITBON M. 1981 Production of haploid Gerbera jamesonii plants by *in vitro* culture of unfertilized ovules. Agronomie 1, 807-812.

SKIRM G.W. 1942 Embryo culturing as an aid to plant breeding. J. Hered. 33, 211-215.

SOMERS D.A., GEGENBACH B.G., BIESBOER D.D., HACKETT W.P. & GREEN C.E. (eds.) 1986 Abstracts VI Int. Cong. Plant Tissue and Cell Culture. Internat. Assoc. Plant Tiss. Cult. Minneapolis, Minn.

STEWARD F.C. & SHANTZ E.M. 1956 The chemical induction of growth in plant tissue cultures. pp. 165-186 in Wain and Wightman (eds.) 1956 *The Chemistry and Mode of Action of Plant Growth Substances.* Butterworth Scientific Publications Ltd., London.

STEWART J. & BUTTON J. 1978 Development of callus and plantlets from *Epidendrum* root tips cultured *in vitro*. Am. Orchid Soc. Bull. 47, 607-612.

STREET H.E. 1954 Growing roots without plants. Discovery 15, 286-292.

STREET H.E. 1957 Excised root culture. Biol. Rev. 32, 117-155.

STREET H.E. 1969 Growth in organised and unorganised systems - knowledge gained by culture of organs and tissue explants. pp. 3-224 in Steward F. C. (ed.) 1969. *Plant Physiology - a Treatise*. 5B. Academic Press, New York.

STREET H.E. (ed.) 1977a *Plant Tissue and Cell Culture.* Bot. Monographs Vol.11 Blackwell Scientific Publications. Oxford, London.

STREET H.E. 1977b Cell (suspension) cultures - techniques. pp. 61-102 in Street H.E. (ed.) 1977a (q.v.).

STREET H.E. 1977c Single cell clones - derivation and selection. pp. 207-222 in Street H.E. (ed.) 1977a (q.v.).

STUART R. & STREET H.E. 1969 The initiation of division in suspensions of stationary-phase cells of *Acer pseudoplatanus* L. J. Exp. Bot. 20, 556-571.

SUNDERLAND N. & DUNWELL J.M. 1977 Anther and pollen culture. pp. 223-265 in Street H.E. (ed.) 1977a (q.v.).

SUNDERLAND N. 1974 Anther culture as a means of haploid induction. pp. 91-122 in Kasha K.J. (ed.) *Haploids in Higher Plants: Advances and Potential.* University of Guelph, Guelph, Canada.

SUNDERLAND N. 1979 Comparative studies of anther and pollen culture. pp. 203-219 in Sharp *et al.* (eds.) 1979 (q.v.).

TAKEBE I., LABIB G. & MELCHERS G. 1971 Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwissenschaften 58, 318-320.

TAKEBE I., OTSUKI Y. & AOKI S. 1968 Isolation of tobacco mesophyll cells in intact and active state. Plant Cell Physiol. 9, 115-124.

TEISSON C. & ALVARD D 1995 A new concept of plant *in vitro* cultivation in liquid medium: temporary immersion. pp. 105-109 in Terzi B., Cella R. & Falavigna A. (eds.). *Current issues in plant molecular and cell biology.* Academic Publishers, Dordrecht.

THENGANE S., PARANJPE S.V., KHUSPE S.S. & MASCARENHAS A.F. 1986 Hybridization of *Gossypium* species through in ovulo embryo culture. Plant Cell Tissue Organ Cult. 6, 209-219.

THOMAS E. & STREET H.E. 1972 Factors influencing morphogenesis in excised roots and suspension cultures of *Atropa belladonna*. Ann. Bot. 36, 239-247.

THORPE T.A. (ed.) 1978 *Frontiers of Plant Tissue Culture*. Int. Assoc. for Plant Tissue Culture. Distr. By the Bookstore, Univ. of Calgary, Calgary Alberta, T2N 1N4, Canada.

THORPE T.A. (ed.) 1981 *Plant Tissue Culture. Methods and Applications in Agriculture.* Academic Press, New York, London, Toronto, Sydney.

TOMES D.T, ELLIS B.E., HARNEY P.M., KASHA K.J. & PETERSON R.L. (eds.) 1982 *Application of Plant Cell and Tissue Culture to Agriculture and Industry.* Plant Cell Culture Centre, Univ. of Guelph, Ontario, Canada.

TORREY J.G. & REINERT J. 1961 Suspension cultures of higher plant cells in synthetic media. Plant Physiol. 36, 483-491.

TORREY J.G. 1958 Endogenous bud and root formation by isolated root of *Convolvulus* grown *in vitro*. Plant Physiol. 33, 258-263.

TORREY J.G. 1963 Cellular patterns in developing roots. pp. 285-314 in Symp. XVII Soc. Exp. Biol., Cambridge University Press.

TORREY J.G. 1973 Plant embryo. pp. 166-170 in Kruse and Patterson (eds.) 1973 (q.v.).

TRUONG-ANDRE I. & DEMARLY Y. 1984 Obtaining plants by *in vitro* culture of unfertilised maize ovaries (*Zea mays* L.) and preliminary studies on the progeny of a gynogenetic plant. Z. Pflanzenzucht. 92, 309-320.

TULECKE W.R. 1953. A tissue derived from pollen of *Ginkgo biloba*. Science 117, 599-600.

TUKEY H.B. 1934 Artificial culture methods for isolated embryos of deciduous fruits. Proc. Am. Soc. Hort. Sci. 32, 313-322.

VASIL I.K. & HILDEBRANDT A.C. 1966 Variations of morphogenetic behaviour in plant tissue cultures. I. *Cichorium endivia*. Am. J. Bot. 53, 860-869.

VASIL I.K. & NITSCH C. 1975 Experimental production of pollen haploids and their uses. Z. Pflanzenphysiol. 76, 191-212.

VASIL I.K. (ed.) 1980a *Perspectives in Plant Cell and Tissue Culture*. Int. Rev. Cytology Suppl. 11A. Academic Press.

VASIL I.K (ed.) 1980b *Perspectives in Plant Cell and Tissue Culture.* Int. Rev. Cytology Suppl. 11B. Academic Press.

VASIL I.K. 1980c Androgenetic haploids. pp. 195-223 in Vasil I.K. (ed.) 1980a (q.v.).

WEATHERS P. & GILES K.L. 1987 Regeneration of many varieties of plants using a novel method of *in vitro* cell culture. In Vitro Cell Dev. Biol. 23, 69A (Abst.).

WETTER L.R. & CONSTABEL F. (eds.) 1982 *Plant Tissue Culture Methods.* (2nd ed.). National Res. Council of Canada. Prairie Regional Lab., Saskatoon.

WHITE P.R. 1954 *The Cultivation of Animal and Plant Cells*. 1st. edition. Ronald Press, New York.

XU C.H., BLAIR L.C., ROGERS S.M.D., GOVINDJEE & WIDHOLM J.M. 1988 Characteristics of five new photoautotrophic suspension cultures including two *Amaranthus* species and a cotton strain growing on ambient CO_2 levels. Plant Physiol. 88, 1297-1302.

YANG H.Y. & ZHOU C. 1990 *In vitro* gynogenesis. in Bhojwani S.S. (ed.) 1990 *Plant Tissue Culture. Applications and Limitations.* Elsevier Science Publishers.

YEOMAN M.M. & FORCHE E. 1980 Cell proliferation and growth in callus cultures. pp. 1-24 in Vasil I.K. (ed.) 1980a (q.v.).

YEOMAN M.M. & STREET H.E. 1977 General cytology of cultured cells. pp. 137-176 in Street H.E. (ed.) 1977a (q.v.).

YEUNG E. C., THORPE T. A. & JENSEN C. J. 1981 *In vitro* fertilization and embryo culture. pp. 253-271 in Thorpe T. A. (ed.) 1981 (q.v.).

ZAITLIN M. 1959 Isolation of tobacco leaf cells capable of supporting virus multiplication. Nature 184, 1002-1003.

ZAPATA F.J. & SINK K.C. 1980 Somatic embryogenesis in *Lycopersicon peruvianum* leaf protoplasts. HortScience 15, 415 (Abst. 313).

ZELCER A., SOFERMAN O. & IZHAR S. 1983 Shoot regeneration in root cultures of Solanaceae. Plant Cell Rep. 2, 252-254.

ZENKTELER M. 1980 Intraovarian and *in vitro* pollination. pp. 137-156 in Vasil I.K. (ed.) 1980b (q.v.).

ZILIS M.R. & MEYER M.M. 1976 Rapid *in vitro* germination of immature, dormant embryos. Comb. Proc. Int. Plant Prop. Soc. 26, 272-2.

Chapter 2
Micropropagation: Uses and Methods

1. SEED VERSUS SOMA

Plants can be propagated through their two developmental life cycles; the sexual, or the asexual. In the sexual cycle new plants arise after fusion of the parental gametes, and develop from zygotic embryos contained within seeds or fruits. In most cases seedlings will be variable and each one will represent a new combination of genes, brought about during the formation of gametes (meiotic cell division) and their sexual fusion. By contrast, in the vegetative (asexual) cycle the unique characteristics of any individual plant selected for propagation (termed the mother plant, stock plant or ortet) are usually perpetuated because, during normal cell division (mitosis), genes are typically copied exactly at each (mitotic) division. In most cases, each new plant (or ramet) produced by this method may be considered to be an extension of the somatic cell line of one (sexually produced or mutant) individual. A group of such asexually reproduced plants (ramets) is termed a clone. In the natural environment sexual and asexual reproduction have their appropriate selective advantages according to the stage of evolution of different kinds of plants. Plants selected and exploited by man also have different propensities for propagation by seed or by vegetative means.

1.1. PROPAGATION USING SEEDS

Seeds have several advantages as a means of propagation:

- they are often produced in large numbers so that the plants regenerated from them are individually inexpensive;
- many may usually be stored for long periods without loss of viability;
- they are easily distributed;
- most often plants grown from seed are without most of the pests and diseases which may have afflicted their parents.

For many agricultural and horticultural purposes it is desirable to cultivate clones or populations of plants which are practically identical. However, the seeds of many plants typically produce plants which differ genetically, and to obtain seeds which will give uniform offspring is either very difficult, or impossible in practical terms. Genetically uniform populations of plants can result from seeds in three ways:

- from inbred (homozygous) lines which can be obtained in self-fertile (autogamous) species. Examples of autogamous crops are wheat, barley, rice and tobacco.
- from F1 seeds produced by crossing two homozygous parents. Besides being uniform, F1 plants may also display hybrid vigour. F1 seeds of many flower producing ornamentals and vegetables are now available, but due to high production costs, they are expensive.
- from apomictic seedlings. In a few genera, plants that are genotypically identical to their parents are produced by apomixis. Seeds are formed without fertilisation and their embryos develop by one of several asexual processes that ensure that the new plants are genetically identical to the female parent (i.e. they have been vegetatively reproduced) (reviewed by Van Dijk and Van Damme, 2000).

Some plants do not produce viable seeds, or do so only after a long juvenile period. Alternatively, to grow plants from seed may not provide a practical method of making new field plantings. In such instances vegetative propagation is the only means of perpetuating and multiplying a unique individual with desirable characteristics.

1.2. VEGETATIVE PROPAGATION

Many important crop plants are increased vegetatively and grown as clones. They include cassava, potato, sugar cane and many soft (small) fruits and fruit trees . A very large number of herbaceous and woody ornamental plants are also propagated by these means. Suitable methods for vegetative propagation have been developed over many centuries. These traditional 'macro-propagation' techniques (or 'macro-methods') which utilise relatively large pieces of plants, have been refined and improved by modern horticultural research. For instance, methods of applying fine water mist to prevent the desiccation of cuttings, better rooting composts and the control of temperature in the rooting zone, have considerably enhanced the rate at which many plants of horticultural or agricultural interest can be multiplied.

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Research to improve macropropagation methods continues, but has lost some impetus in recent years with the continued extension of tissue culture for plant multiplication.

Whether it will be most rewarding to propagate a plant by seed, by traditional vegetative techniques, or by tissue culture, will often not only depend on the plant species, but also on the development of proven techniques, relative costs and agronomic objectives. The extent to which tissue culture methods can be used for genetic manipulations and for propagation is changing continuously. Until recently, potato plants have been raised from seed during breeding programmes to select new varieties: tissue culture may have been employed to multiply certain lines and to propagate disease-tested stocks of established cultivars, while macropropagation of field-grown tubers has been used to provide normal planting material. New research into genetic manipulations and methods of propagation using tissue culture techniques, can alter this situation: diversity can be introduced and controlled through genetic engineering while certified stock of new varieties can be produced on a large scale by micropropagation.

The selection of a propagation method for any given plant is constrained by its genetic potential. For example, some plants readily produce adventitious shoot buds on their roots, while others do not; trying to propagate a plant, which does not have this capability, from root cuttings or root explants, will be more problematic both *in vivo* and *in vitro*. Plant tissue culture does overcome some genetically imposed barriers, but a clear effect of genotype is still apparent. It is not yet possible to induce an apple tree to produce tubers!

2. PROPAGATION *IN VITRO*

2.1. ADVANTAGES

Methods available for propagating plants *in vitro* are largely an extension of those already developed for conventional propagation. *In vitro* techniques have the following advantages over traditional methods:

- Cultures are started with very small pieces of plants (explants), and thereafter small shoots or embryos are propagated (hence the term ‘micropropagation’ to describe the *in vitro* methods). Only a small amount of space is required to maintain plants or to greatly increase their number. Propagation is ideally carried out in aseptic conditions (avoiding contaminations). The often used term “axenic” is not correct, because it means “free from any association with other living organisms”. Once cultures have been started there should be no loss through disease, and the plantlets finally produced should be ideally free from bacteria, fungi and other micro-organisms. (Most often this is not the case, see Vol. 2).
- Methods are available to free plants from specific virus diseases. Providing these techniques are employed, or virus-tested material is used for initiating cultures, certified virus-tested plants can be produced in large numbers. Terminology such as virus-free and bacteria-free should not be used, as it is impossible to prove that a plant is free of all bacteria or viruses. One can only prove that a plant has been freed from a specific contaminant provided the appropriate diagnostic tools are available.
- A more flexible adjustment of factors influencing vegetative regeneration is possible such as nutrient and growth regulator levels, light and temperature. The rate of propagation is therefore much greater than in macropropagation and many more plants can be produced in a given time. This may enable newly selected varieties to be made available quickly and widely, and numerous plants to be produced in a short while. The technique is very suitable when high volume production is essential.
- It may be possible to produce clones of some kinds of plants that are otherwise slow and difficult (or even impossible) to propagate vegetatively.
- Plants may acquire a new temporary characteristic through micropropagation which makes them more desirable to the grower than conventionally-raised stock. A bushy habit (in ornamental pot plants) and increased runner formation (strawberries) are two examples.
- Production can be continued all the year round and is more independent of seasonal changes.
- Vegetatively-reproduced material can often be stored over long periods.
- Less energy and space are required for propagation purposes and for the maintenance of stock plants (ortets).
- Plant material needs little attention between subcultures and there is no labour or materials requirement for watering, weeding, spraying etc.; micropropagation is most advantageous when it costs less than traditional methods of multiplication; if this

is not the case there must be some other important reason to make it worthwhile.

2.2. DISADVANTAGES

The chief disadvantages of *in vitro* methods are that advanced skills are required for their successful operation.

- A specialised and expensive production facility is needed; fairly specific methods may be necessary to obtain optimum results from each species and variety and, because present methods are labour intensive, the cost of propagules is usually relatively high (Vol. 2). Further consequences of using *in vitro* adaptations are although they may be produced in large numbers, the plantlets obtained are initially small and sometimes have undesirable characteristics.
- In order to survive *in vitro*, explants and cultures have to be grown on a medium containing sucrose or some other carbon source. The plants derived from these cultures are not initially able to produce their own requirement of organic matter by photosynthesis (i.e. they are not autotrophic) and have to undergo a transitional period before they are capable of independent growth. More recently techniques have been proposed which allow the production of photo-autotrophic plants *in vitro* (Kozai and Smith, 1995).
- As they are raised within glass or plastic vessels in a high relative humidity, and are not usually photosynthetically self-sufficient, the young plantlets are more susceptible to water loss in an external environment. They may therefore have to be hardened in an atmosphere of slowly decreasing humidity and increased light. The chances of producing genetically aberrant plants may be increased.

A more extended discussion of all these points will be found in other sections.

2.3. TECHNIQUES

The methods that are theoretically available for the propagation of plants *in vitro* are illustrated in Fig 2.1 and described in the following sections of this Chapter. They are essentially:

- by the multiplication of shoots from axillary buds:
- by the formation of adventitious shoots, and/or adventitious somatic embryos, either a) directly on pieces of tissue or organs (explants) removed from the mother plant; or b) indirectly from unorganised cells (in suspension cultures) or tissues (in callus cultures) established by the proliferation of cells within explants; on semi-organised callus tissues or propagation bodies (such as protocorms or pseudo-bulbils) that can be obtained from explants (particularly those from certain specialised whole plant organs).

The techniques that have been developed for micropropagation are described in greater detail in the following sections of this Chapter. In practice most micropropagated plants are produced at present by method (i), and those of only a few species (which will be instanced later) by method (ii). Shoots and/or plantlets do not always originate in a culture by a single method. For example, in shoot cultures, besides axillary shoots, there are sometimes adventitious shoots formed directly on existing leaves or stems, and/or shoots arising indirectly from callus at the base of the explant. The most suitable and economic method for propagating plants of a particular species could well change with time. There are still severe limitations on the extent to which some methods can be used. Improvements will come from a better understanding of the factors controlling morphogenesis and genetic stability *in vitro*.

Rooting Somatic embryos have both a root and a shoot meristem. Under ideal conditions they can grow into normal seedlings. The shoots procured from axillary or adventitious meristems are miniature cuttings. Sometimes these small cuttings form roots spontaneously, but usually they have to be assisted to do so (Fig 2.2). The small rooted shoots produced by micropropagation are often called plantlets.

2.4. STAGES OF MICROPROPAGATION

Professor Murashige of the University of California (Riverside) defined three steps or stages (I-III) in the *in vitro* multiplication of plants (Murashige, 1974). These have been widely adopted by both research and commercial tissue culture laboratories because they not only describe procedural steps in the micropropagation process, but also usually represent points at which the cultural environment needs to be changed.

Some workers have suggested that the treatment and preparation of stock plants should be regarded as a separately numbered stage or stages. We have adopted the proposal of Debergh and Maene (1981) that such preparative procedures should be called Stage 0. A fourth stage (IV), at which plants are transferred to the external environment, is now also commonly recognised. A general description of Stages 0-IV is therefore provided below, while the manner in which Stages I-III might be applied to different methods of micropropagation is given in Table 2.1.

Table 2.1 Stages in the available methods of micropropagation.

Methods of Micropropagation	Stage of culture		
	I. Initiating a culture Growth of excited tissues/organs *in vitro* free from algae, bacteria, fungi and other contaminants.	**II.** Increasing propagules Inducing the cultures to produce numbers of shoots or somatic embryos.	**III.** Preparation for soil transfer Separating and preparing propagules to have a high rate of survival as individual plants in the external environment.
Shoot Cultures	Transfer of disinfected shoot tips or lateral buds to solid or liquid media and the commencement of shoot growth to *ca*. 10mm.	Induce multiple (axillary) shoot formation and growth of the shoots to a sufficient size for separation, either as new Stage II explants or for passage to III.	Elongation of buds formed at Stage II to uniform shoots. Rooting the shoots *in vitro* or outside the culture vessel.
Shoots from floral meristems	Aseptic isolation of pieces of compound floral meristems.	Inducing the many meristems to produce vegetative shoots, then as shoot tip culture.	As for shoot tip cultures.
Multiple shoots from seeds	Aseptic germination of seeds on a high cytokinin medium.	Inducing multiple shoot proliferation. Shoot subculture	As for shoot tip cultures.
Meristem culture	Transfer of very small shoot tips (length 0.2-0.5mm) to culture. Longer shoot tips (1-2mm) can be used as explants if obtained from heat treated plants.	Growth of shoots to *ca.* 10mm, then as shoot tip culture, or as shoot multiplication omitted and shoots transferred to Stage III.	As for shoot tip cultures.
Node culture	As for shoot tip culture but shoots grown longer to show clear internodes.	Propagation by inducing the axillary bud at each node to grow into a single shoot. Subculturing can be repeated indefinitely.	As for shoot tip cultures.
Direct shoot regeneration from explants	Establishing suitable explants of mother plant tissue (e.g. leaf or stem segments) in culture without contamination.	The induction of shoots directly on the explant with no prior formation of callus. Shoots so formed can usually be divided and used as explants for new Stage II subcultures or shoot tip culture.	As for shoot tip cultures.
Direct embryogenesis	Establishing suitable embryogenic tissue explants or previously-formed somatic embryos.	The direct induction of somatic embryos on the explants without prior formation of callus.	Growth of the embryos into plantlets which can be transferred to the outside environment.
Indirect shoot regeneration from morphogenic callus	Initiation and isolation of callus with superficial shoot meristems.	Repeated subculture of small callus pieces followed by transfer to a shoot-inducing medium. The growth of shoots *ca*. 10mm in length.	Individual shoots are grown and rooted.
Indirect embryogenesis from embryogenetic callus or suspension cultures	Initiation and isolation of callus with the capacity to form somatic embryos, OR obtaining embryogenic suspension cultures from embryogenic callus or by *de novo* induction.	Subculture of the embryogenic callus or suspension culture followed by transfer to a medium favouring embryo development.	Growth of the somatic embryos into “Seedlings”.
Storage organ formation	Isolation and culture of tissue/organs capable of forming storage organs.	Inducing the formation of storage organs and sometimes dividing them to start new Stage II cultures.	Growing shoots/plantlets obtained from storage organs for transfer to soil: OR growing the storage organs themselves to a size suitable for soil planting.

Requirements for the completion of each stage of micropropagation vary according to the method being utilised; the progress of cultures will not always fit readily into neat compartments. Furthermore, it is not always necessary to follow each of the prescribed steps. The stages are therefore described here for general guidance but should not be applied too rigidly.

Practices adopted at the various stages of micropropagation are discussed throughout the book, but are particularly mentioned in Volume 2.

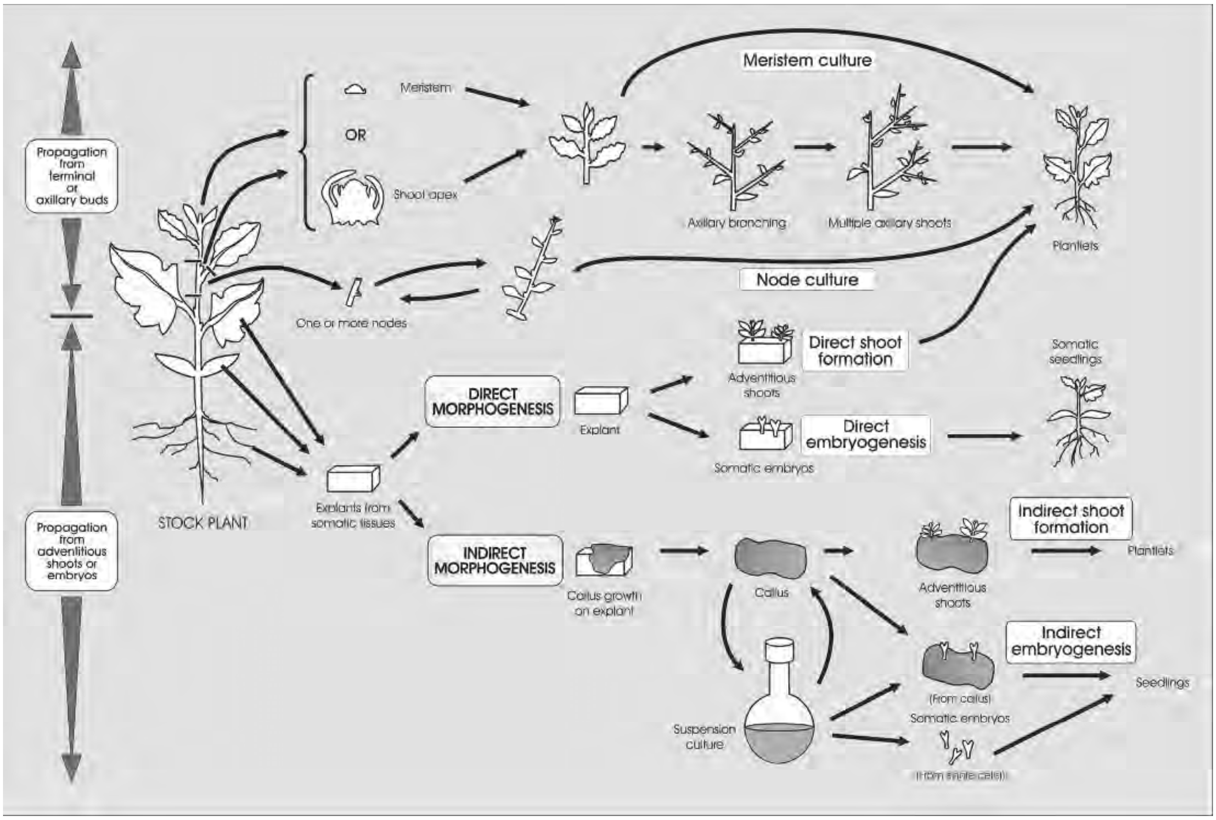

Fig. 2.1 The principal methods of micropropagation.

Stage 0: Mother plant selection and preparation

Before micropropagation commences, careful attention should be given to the selection of stock plants. They must be typical of the variety or species, and free from any symptoms of disease. It may be advantageous to treat the chosen plant (or parts of it) in some way to make *in vitro* culture successful. Steps to reduce the contamination level of explants (Vol. 2) were considered sufficiently important by Debergh and Maene (1981) to constitute a separate essential stage in a commercial micropropagation programme. Growth, morphogenesis and rates of propagation *in vitro* can be improved by appropriate environmental and chemical pre-treatment of stock plants: this subject is discussed in Chapter 11. Procedures to detect and reduce or eliminate systemic bacterial and virus diseases (see Vol. 2) may also be required. Disease indexing and disease elimination should be a definite part of all micropropagation work; but these precautions are unfortunately often omitted, sometimes with adverse consequences. The difficulties which may be encountered in trying to propagate chimeras by tissue culture methods are discussed in Chapter 10.

It seems appropriate to include all procedures adopted in plant selection and pre-treatment within ‘Stage 0’. The recognised numbering of Murashige's stages is then unaltered.

Stage I: Establishing an aseptic culture

The customary second step in the micropropagation process is to obtain an aseptic culture of the selected plant material. Success at this stage firstly requires that explants should be

transferred to the cultural environment, free from obvious microbial contaminants; and that this should be followed by some kind of growth (e.g. growth of a shoot tip, or formation of callus). Usually a batch of explants is transferred to culture at the same time. After a short period of incubation, any container found to have contaminated explants or medium is discarded. Stage I would be regarded as satisfactorily completed if an adequate number of explants had survived without contamination, and was growing on. The objective is reproducibility, not 100% success.

Stage II: The production of suitable propagules

The object of Stage II is to bring about the production of new plant outgrowths or propagules, which, when separated from the culture are capable of giving rise to complete plants. According to the *in vitro* procedure that is being followed (Fig 2.1), multiplication can be brought about from newly-derived axillary or adventitious shoots, somatic embryos, or miniature storage or propagative organs. In some micropropagation methods, Stage II will include the prior induction of meristematic centres from which adventitious organs may develop. Some of the propagules produced at Stage II (especially shoots) can also be used as the basis for further cycles of multiplication in that they can usually be cultured again (subcultured) to increase their number.

Stage III: Preparation for growth in the natural environment

Shoots or plantlets derived from Stage II are small, and not yet capable of self-supporting growth in soil or compost. At Stage III, steps are taken to grow individual or clusters of plantlets, capable of carrying out photosynthesis, and survival without an artificial supply of carbohydrate. Some plantlets need to be specially treated at this stage so that they do not become stunted or dormant when taken out of the cultural environment. As originally proposed by Murashige, Stage III includes the *in vitro* rooting of shoots prior to their transfer to soil.

Rooting shoots is a very important part of any *in vitro* propagation scheme. A few species form adventitious roots on shoots during the course of Stage III culture, but usually it is necessary to adopt a separate rooting procedure using special media, or methods, to induce roots to form. Sometimes shoots may need to be specially elongated before rooting is attempted. To reduce the costs of micropropagation, many laboratories now remove unrooted shoots from the *in vitro* environment and root them outside the culture vessel (Fig 2.2). Therefore, in cultures where micropropagation relies on adventitious or axillary shoots, Stage III is often conveniently divided, as Debergh and Maene (1981) suggested, into:

- Stage IIIa, the elongation of buds or shoots formed during Stage II, to provide shoots of a suitable size for Stage IIIb;
- Stage IIIb, the rooting of Stage IIIa shoots *in vitro* or *extra vitrum*.

Procedures used to induce rooting are discussed in later chapters.

Stage IV: Transfer to the natural environment

Although not given a special numerical stage by Murashige, the methods whereby plantlets are transferred from the *in vitro* to the *ex vitro* external environment are extremely important. If not carried out carefully, transfer can result in significant loss of propagated material. There are two main reasons:
—Shoots developed in culture have often been produced in high humidity and a low light ‘intensity’. This results in there being less leaf epicuticular wax or wax with an altered chemical composition, than on plants raised in growth chambers or greenhouses. In some plants, the stomata of leaves produced *in vitro* may also be atypical and incapable of complete closure under conditions of low relative humidity. Tissue cultured plants therefore lose water rapidly when moved to external conditions (Sutter and Langhans, 1979, 1980).
—When supplied with sucrose (or some other carbohydrate) and kept in low light conditions, micropropagated plantlets are not fully dependent on their own photosynthesis (they are mixotrophic - Chapter 12). A stimulus which is not provided in the closed *in vitro* environment seems to be needed for them to change to being fully capable of producing their own requirements of carbon and reduced nitrogen (i.e. before they become capable of feeding themselves - autotrophic) (Marin and Gella, 1987). The change only occurs after the plants have spent a period of several days *ex vitro*.

In practice, plantlets are removed from their Stage III containers, and if they have been grown on agar medium, the gel is carefully washed from the roots. The application of an anti-transpirant film to the leaves has been recommended at this stage, but in practice, seems to be seldom used. Plantlets are then transplanted into an adequate rooting medium (such as a peat:sand compost) and kept for several days in high humidity and reduced light intensity. A fog of water vapour is very effective for maintaining humidity. Alternatively, intermittent water misting may be applied automatically, or the plants placed

inside a clear plastic enclosure and misted by hand. With some plants, an *in vitro* Stage III can be omitted; shoots from Stage II are rooted directly in high humidity, and, at the same time, gradually hardened to the exterior environment.

The rooting of shoots using these methods is discussed fully in Volume 2.

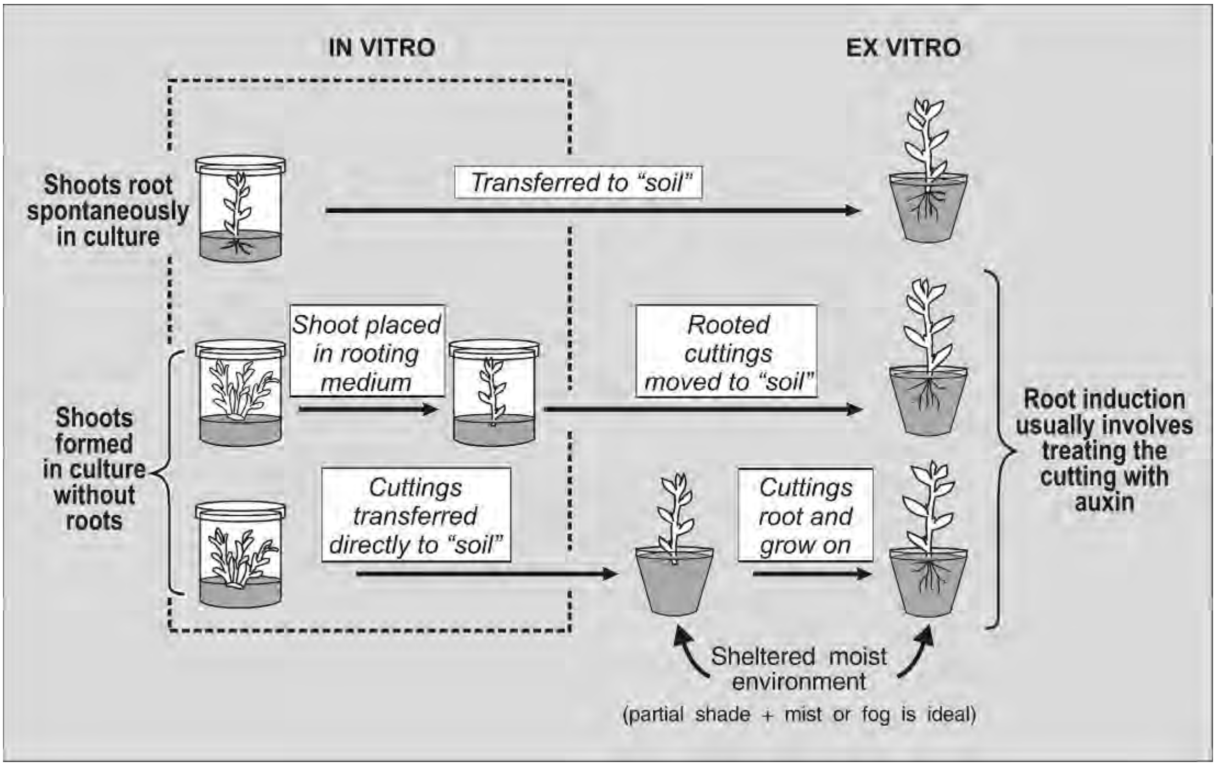

Fig. 2.2 Alternative methods of rooting micropropagated shoots.

3. MICROPROPAGATION METHODS

3.1. THE PROPAGATION OF PLANTS FROM AXILLARY BUDS OR SHOOTS

The production of plants from axillary buds or shoots has proved to be the most generally applicable and reliable method of true-to-type *in vitro* propagation. Two methods are commonly used:

- Shoot culture
- Single, or multiple, node culture.

Both depend on stimulating precocious axillary shoot growth by overcoming the dominance of shoot apical meristems.

3.1.1. Shoot (or shoot tip) culture

The term shoot culture is now preferred for cultures started from explants bearing an intact shoot meristem, whose purpose is shoot multiplication by the repeated formation of axillary branches. In this technique, newly formed shoots or shoot bases serve as explants for repeated proliferation; severed shoots (or shoot clumps) are finally rooted to form plantlets which can be grown *in vivo*. This is the most widely used method of micropropagation.

Explant size. Shoot cultures are conventionally started from the apices of lateral or main shoots, up to 20 mm in length, dissected from actively-growing shoots or dormant buds. Larger explants are also sometimes used with advantage: they may consist of a larger part of the shoot apex or be stem segments bearing one or more lateral buds; sometimes shoots from other *in vitro* cultures are employed. When apical or lateral buds were used almost exclusively as explants, the name 'shoot tip culture' came to be widely used for cultures of this kind. As the use of larger explants has become more common, the term shoot culture has become more appropriate.

Large explants have advantages over smaller ones for initiating shoot cultures in that they:

- better survive the transfer to *in vitro* conditions
- more rapidly commence growth
- contain more axillary buds

However, the greater the size of the explant, the more difficult it may be to decontaminate from micro-organisms; in practice the size used will be the largest that can be gained in aseptic conditions. Shoot cultures are also frequently started directly from the shoots obtained from meristem tip cultures. Virus eradication then proceeds the shoot multiplication phase. Occasionally fragmented or macerated shoot tips are used (see elsewhere in this Chapter). Meristem tip or meristem cultures are used for virus and bacteria elimination. Meristem cultures are initiated from much smaller explants and a single plantlet is usually produced from each. This terminology is very often abused.

Regulating shoot proliferation

The growth and proliferation of axillary shoots in shoot cultures is usually promoted by incorporating growth regulators (usually cytokinins) into the growth medium. Most often such a treatment effectively removes the dominance of apical meristems so that axillary shoots are produced, often in large numbers. These shoots are used as miniature cuttings for plant multiplication.

Removing the apex. In some plants, pinching out the main shoot axis is used as an alternative, or an adjunct, to the use of growth regulators for decreasing apical dominance. Pinching was found to be effective for some kinds of rose (Bressan *et al.,* 1982) and for some apple cultivars (Yae *et al.,* 1987). Pinching or ‘tipping’ is usually done when plant material is removed for subculturing, for example removing the apical bud at the first subculture increased the branching of *Pistacia* shoot cultures (Barghchi, 1986). An effective kind of shoot tipping occurs when shoots are cropped as microcuttings. Standardi (1982) and Shen and Mullins (1984) obtained effective shoot proliferation of kiwi and pear varieties by transferring the basal shoot clump that is left at this stage, to fresh medium for further proliferation. (Note however that this practice can increase the likelihood of obtaining deviant plants - see below). In just a few plants neither cytokinins nor pinching effectively remove apical dominance. Geneve *et al.* (1990) reported that seedling shoots of *Gymnocladus dioicus* produced 1–5 shoots, but only one grew to any appreciable length. If this shoot was removed, another took over.

Placing explants horizontally. In pear, pinching out the tips of shoots resulted in the growth of larger axillary shoots than in the controls, but the number of shoots was less. The most effective physical check to apical dominance was achieved by pinching the tips, and/or placing shoot explants horizontally on the medium (Lane, 1979; MacKay and Kitto, 1988). The treatment can be effective with many other woody plants: horizontal placement of shoot sections, consisting of 2-3 nodes, resulted in more axillary shoots being produced in cultures of *Acer rubrum, Amelanchier spicata*, *Betula nigra, Forsythia intermedia* and *Malus domestica*, than when explants were upright (McClelland and Smith, 1990). Favourable results have also been reported with lilac (Hildebrandt and Harney, 1983) and some apple cultivars (Yae *et al.,* 1987).

The origin of shoots

Unfortunately not all the shoots arising in shoot cultures may originate from axillary buds. Frequently, adventitious shoots also arise, either directly from cultured shoot material, or indirectly from callus at the base of the subcultured shoot mass. For example, Nasir and Miles (1981) observed that in subcultures of an apple rootstock, some new shoots arose from callus at the base of the shoot clump; both adventitious and axillary shoots were produced in *Hosta* cultures (Papachatzi *et al.,* 1981); and shoot proliferation from some kinds of potato shoot tips was exclusively from organogenic callus (Roca *et al.,* 1978).

The precise origin of shoots can sometimes only be determined from a careful anatomical examination. Hussey (1983) has termed cultures providing both adventitious and axillary shoots, ‘mixed cultures’. Adventitious shoots, particularly those arising indirectly from callus, are not desirable. For reasons described in Volume 2, shoots of axillary origin will normally be genetically identical to the parent plant, whereas there is a probability that those regenerated from callus may differ in one or more characters. Genetically deviant plants may not occur with high frequency from newly initiated callus, but could begin to appear in significant number if shoot masses incorporating basal callus are simply chopped up to provide explants for subculture. The use of a strict protocol, using only axillary shoots, may present problems with some plants where the rate of shoot multiplication is comparatively slow. This has led to attempts by some workers to use a more relaxed regime and accept a proportion of

adventitious shoots (e.g. with *Kalanchoe blossfeldiana* - Schwaiger and Horn, 1988). The usual consequence is a degree of variation amongst ramets which may, or may not, be acceptable. The formation of callus and the subsequent development of adventitious shoots can often be controlled by modifying the growth regulators in the medium.

Fragmentation of a meristem tip, or its culture in a certain way, can lead to the formation of multiple adventitious shoots which can be used for plant propagation. These modifications of conventional shoot culture are described on in Chapter 10.

History

Although shoot culture has proved to be a widely applicable method of micropropagation, the appreciation of its potential value developed only slowly, and utilisation largely depended on improvements in tissue culture technology. Robbins (1922) seems to have been the first person to have successfully cultured excised shoot tips on a medium containing sugar. Tip explants of between 1.75 and 3.75 mm were taken from pea, corn and cotton, and placed in a liquid medium. For some reason the cultures were maintained in the dark where they only produced shoots with small chlorotic leaves and numerous roots. Although it is tempting to suppose that the potential of shoot culture for plant propagation might have been appreciated at a much earlier date had the cultures been transferred to the light, the rapid rate of shoot multiplication achieved in modern use of this technique depends on later developments in plant science.

Only very slow progress in shoot culture was made during the next 20 years. As part of his pioneering work on plant tissue culture, White (1933) experimented with small meristem tips (0.1 mm or less) of chickweed (*Stellaria media*), but they were only maintained in hanging drops of nutrient solution. Leaf or flower primordia were observed to develop over a six-week period. Shoot culture of a kind was also carried out by La Rue (1936). His explants largely consisted of the basal and upper halves of seed embryos. Nevertheless, the apical plumular meristems of several plants were grown to produce entire plants. Whole plants were also obtained from axillary buds of the aquatic plant *Radicula aquatica*.

Significant shoot growth from vegetative shoot tip explants was first achieved by Loo, and reported in 1945 and 1946 a, b. *Asparagus* shoot tips 5-10 mm in length were supported on glass wool over a liquid medium and later grown on a solidified substrate. Loo (1945, 1946a, 1946b) made several significant observations showing that:

- growth depended on sucrose concentration, higher levels being necessary in the dark than in the light;
- explants, instead of being supported, could be grown satisfactorily on 0.5% agar;
- *in vitro* shoot growth could apparently be continued indefinitely (35 transfers were made over 22 months);
- shoot tip culture afforded a way to propagate plant material (clones were established from several excised shoot apices).

This work failed to progress further because no roots were formed on the *Asparagus* shoots in culture. Honours for establishing the principles of modern shoot culture must therefore be shared between Loo and Ball. Ball (1946) was the first person to produce rooted shoots from cultured shoot apices. His explants consisted of an apical meristem and 2–3 leaf primordia. There was no shoot multiplication but plantlets of nasturtium (*Tropaeolum majus*) and white lupine (*Lupinus alba*) were transferred to soil and grown successfully.

During several subsequent years, shoot apex (or meristem tip) culture was of interest only to plant pathologists who recognised its value for producing virus-tested plants. It was during studies of this kind that Morel made the significant discovery of protocorm formation from *Cymbidium* orchid shoot tips. Although they may be started from the same explants, cultures giving rise to protocorms are not typical shoot cultures (see later in this chapter).

The two major developments which made shoot culture feasible were the development of improved media for plant tissue culture (Murashige and Skoog, 1962) and the discovery of the cytokinins as a class of plant growth regulators (Miller, 1961b; Skoog *et al.,* 1965), with an ability to release lateral buds from dormancy (Wickson and Thimann, 1958; Sachs and Thimann, 1964). These developments were not immediately applied to shoot culture, and some years elapsed before it was appreciated that multiple shoots could be induced to form by appropriate growth regulator treatments.

Hackett and Anderson (1967) got either single shoots from carnation shoot apices, or else a proliferative tissue from which shoots were later regenerated. Walkey and Woolfitt (1968) reported a similar kind of direct or indirect shoot proliferation from *Nicotiana rustica* shoot tips. Vine and Jones (1969) were able to transfer large shoot tips of hop (*Humulus*) to culture, but shoots only rooted, and

showed a high propensity for callus formation. Reports of plant multiplication using conventional shoot culture methods began to appear in the next decade.

Haramaki (1971) described the rapid multiplication of *Gloxinia* by shoot culture and by 1972 several reports of successful micropropagation by this method had appeared (Adams, 1972; Haramaki and Murashige, 1972). Since then the number of papers on shoot culture published annually has increased dramatically and the method has been utilised increasingly for commercial plant propagation. Factors which have influenced the choice of shoot culture for practical micropropagation have been:

- the way in which the method can be applied to a wide range of different plant species, using the same principles and basic methods;
- the possibility of obtaining simultaneous virus control;
- a general uniformity and 'trueness to type' of the regenerated plants;
- the relatively high rates of propagation which is possible in many species.

Methods

Primary explants In most herbaceous plants, shoot tip explants may be derived from either apical or lateral buds of an intact plant, and consist of a meristematic stem apex with a subtended rudimentary stem bearing several leaf initials (Fig 2.3). In the axils of the more developed leaf primordia there will be axillary bud meristems. In some species (e.g. *Eucalyptus*) it is an advantage to commence shoot cultures with a piece of the stem of the mother plant bearing one or more buds (stem nodes). Shoot growth from the bud, and treatment of the culture, is thereafter the same as in conventional shoot tip culture. The use of nodal explants should not be confused with node culture in which a method of shoot multiplication is used that is different to that in shoot culture (see later).

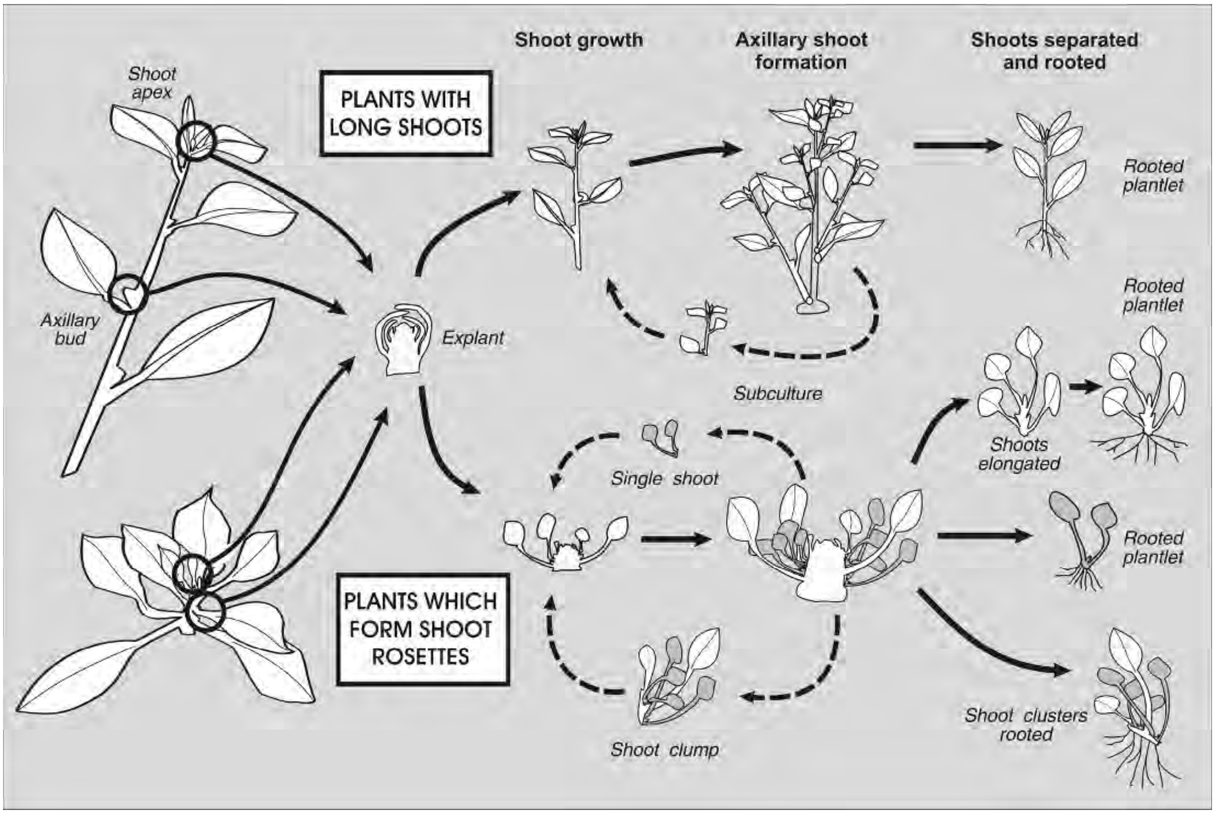

Fig. 2.3 Shoot tip culture.
Separation of axillary shoots for rooting (or subculture) is easier in species which naturally produce long shoots.

Shoot tips from trees, or other woody perennials, can be difficult to decontaminate. Because of this, Standardi and Catalano (1985) preferred to initiate shoot cultures of *Actinidia chinensis* from meristem

tips which could be sterilised more easily. Shoot tips of woody plants are more liable than those of herbaceous species to release undesirable phenolic substances when first placed onto a growth medium. Buds taken from mature parts of the shrub or tree can also be reluctant to grow *in vitro* and seasonal factors may reinforce natural dormancy in buds from any source, so that cultures can only be readily initiated at certain times of the year (see Chapter 11). Shoot tip or lateral bud explants are usually most readily induced into growth if taken from juvenile shoots (see Chapter 11) such as those of seedlings or young plants. The juvenile shoots which sometimes emerge from the base of mature plants or which arise form heavily pruned or coppiced bushes and trees, are alternative sources. However, developing techniques have made it possible to propagate some woody ornamentals, forest trees and fruit trees, using explants derived from mature shoots (see Chapter 11). De Fossard *et al.* (1977) could initiate cultures of *Eucalyptus ficifolia* with shoot tips from 36 year-old trees, but forest-gathered material was very difficult to decontaminate unless covered and protected for some period before excision (stage 0).

Secondary explants. Stage II subcultures are initiated from axillary shoots separated from primary shoot clusters. The place of the secondary explant within the primary shoot (cluster) can have a remarkable influence on the subsequent performance of the subcultures. A higher rate of shoot proliferation is often obtained from nodal explants or by subdivision of the basal shoot mass. Shoot tips were the best secondary explant for *Rosa* 'Fraser McClay', but with cherry ('F12/1') nodal explants gave more than twice as many shoots, and basal masses, three times as many as shoot tips (Hutchinson, 1985). In Sitka spruce, cultures that had been apices in the previous subculture were able to proliferate buds at higher rates than those that had been axillary buds (John and Murray, 1981). The origin of an explant can also have a tremendous influence on the subsequent behaviour of the plant when established under field conditions. This was illustrated by Marks and Meyers (1994) for *Daphne odorata.*

To minimise the risk of genetic change in ramets, explants for subculture and shoots to be transferred to Stage III, should, as far as possible, be chosen from new shoots of axillary origin. It may be advisable to adjust the growth regulator content of the medium so that adventitious shoots are not formed, even though the rate of overall shoot multiplication is thereby reduced. In some circumstances callus arising at the base of an explant may be semi-organised and therefore capable of producing genetically-stable plants (Vol. 2).

Stage II cultures are typically without roots, and shoots need to be detached and treated as miniature cuttings which, when rooted, will provide the new plants that are required. An alternative is to allow shoot clusters to elongate and to root singulated shoots under *ex vitro* conditions (Fig 2.2).

Media and growth regulators Advice on the selection of appropriate media for shoot cultures is given in Volume 2. A notable feature of shoot cultures of most plant species is the need for high cytokinin levels at Stage II to promote the growth of multiple axillary shoots. A description of the compounds which can be employed and effective rates of treatment are given in Chapters 3–7.

Cytokinin growth regulators are usually extremely effective in removing the apical dominance of shoots. Their use can be combined with pinching the apex of shoots, or placing explants in an horizontal position (Chapter 6). A cytokinin treatment can not only promote the formation of multiple shoots (axillary and/or adventitious), but also (if the compound used is unsuitable, or the concentration used is too high), cause the shoots formed to be too short for rooting and transfer.

Because or their nature, or the absence of an adequate method of culture, plants of some kinds fail to produce multiple shoots at Stage II and retain their apical dominance. In shoot cultures of *Gymnocladus dioicus*, for example, despite the formation of several axillary shoots in the presence of BA cytokinin, one shoot nearly always became dominant over the others (Geneve *et al.,* 1990). Most plants of this kind are best propagated by node culture (see below).

Elongation

The length of the axillary shoots produced in shoot cultures varies considerably from one kind of plant to another. Species which have an elongated shoot system *in vivo* will produce axillary shoots which can be easily separated as microcuttings and then individually rooted. Apically dominant shoots which have not branched can be treated in the same way.

At the other extreme are plants with a natural rosette habit of growth, which tend to produce shoot clusters in culture (Fig 2.3). When these are micropropagated, it is difficult to separate individual shoots for use as secondary explants. It may then only be practical to divide the shoot mass into pieces and re-culture the fragments. Such shoot clusters can be

induced to form roots when plants with a bushy habit are required (e.g. many species sold in pots for their attractive foliage). Otherwise it is necessary to specially elongate shoots before they are rooted (Stage IIIa). Shoot clusters are treated in such a way that axillary shoot formation is reduced, and shoot growth promoted. Individual shoots are then more readily handled and can be rooted as microcuttings. Methods for elongating shoots are discussed in Volume 2.

Rooting and transfer The cytokinin growth regulators added to shoot culture media at Stage II to promote axillary shoot growth, usually inhibit root formation. Single shoots or shoot clusters must therefore be moved to a different medium for rooting *in vitro* before being transferred as plantlets to the external environment. An alternative strategy for some plants is to root the plant material *ex vitro*. The methods employed are described in Volume 2. Treatments need to be varied according to the type of growth; the nature of the shoot proliferation produced during Stage II culture; and the plant habit required by the customer.

Current applications Conventional shoot culture continues to be the most important method of micropropagation, although node culture is gaining in importance. It is very widely used by commercial tissue culture laboratories for the propagation of many herbaceous ornamentals and woody plants (see Volume 2 for further details). The large numbers of manipulations required do, however, make the cost of each plantlet produced by this method comparatively expensive. Some success has been achieved in automating some stages of the process, in applying techniques for large-scale multiplication and in the use of robotics for plant separation and planting (Vol. 2).

3.1.2. Shoot proliferation from meristem tips

Barlass and Skene (1978; 1980a,b; 1982a,b) have shown that new shoots can be formed adventitiously when shoot tips of grapevine or *Citrus* are cut into several pieces before culture. Tideman and Hawker (1982) also had success using fragmented apices with *Asclepias rotundifolia* but not with *Euphorbia peplus*. Usually leaf-like structures first develop from the individual fragments; these enlarge and shoots form from basal swellings. Axillary shoots often arise from the initial adventitious shoots.

Shoot cultures transferred to agitated liquid culture may form a proliferating mass of shoots. Although high rates of multiplication are possible, leafy shoots usually become hyperhydric (see Volume 2). However, in some species at least, shoots can be reduced in size to little more than proliferating shoot initials (by adding plant growth retardants, Ziv *et al*,, 1994) which are then suitable for large-scale multiplication (Volume 2). A somewhat similar kind of culture consisting of superficial shoot meristems on a basal callus can sometimes be initiated from shoot tip explants or from the base of shoot cultures (see later in this chapter).

3.1.3. Single and multiple node culture (in vitro layering)

Single node culture is another *in vitro* technique which can be used for propagating some species of plants from axillary buds. As with shoot culture, the primary explant for single node culture is a shoot apex, a lateral bud or a piece of shoot bearing one or more buds (i.e. having one or more nodes). When shoot apices are used, it can be advantageous to initiate cultures with large explants (up to 20 mm), unless virus-tested cultures are required, and small meristem-tips will be employed. Unbranched shoots are grown at Stage I until they are 5–10 cm in length and have several discrete and separated nodes. An environment that promotes etiolated shoot growth may be an advantage. Then at Stage II, instead of inducing axillary shoot growth with growth regulators (as in shoot culture), one of two manipulative methods is used to overcome apical dominance and promote lateral bud break (Fig 2.2):

- intact individual shoots may be placed on a fresh medium in an horizontal position. This method has been used by Wang (1977) to propagate potatoes, and has been termed '*in vitro* layering';
- each shoot may be cut into single-, or several-node pieces which are sub-cultured. Leaves are usually trimmed so that each second stage explant consists of a piece of stem bearing one or more lateral buds.
- each approach can be reiterated to propagate during stage II.

Unfortunately, *in vitro* layering seldom results in several axillary shoots of equal length, as shown in Fig 2.4; apical dominance usually causes the leading shoot, or shoots, to grow more rapidly than the rest. El Hasan and Debergh (1987) found that, even in potato, node culture was preferable. Node culture is therefore the simplest method of *in vitro* propagation, as it requires only that shoot growth should occur. Methods of rooting are the same as those employed for the microcuttings derived from shoot culture, except that prior elongation of shoots is unnecessary.

Note that "node culture" is distinct from shoot cultures started from the nodes of seedlings or mature plants.

Media and growth regulators.

Media for single node culture are intrinsically the same as those suitable for shoot culture. As in shoot culture, optimum growth rate may depend on the selection of a medium particularly suited to the species being propagated, but adequate results can usually be produced from well-known formulations. It is often unnecessary to add growth regulators to the medium; for example, the shoots of some plants (e.g. *Chrysanthemum morifolium*) elongate satisfactorily without any being provided. If they are required, regulants at both Stages I and II will usually comprise an auxin and a cytokinin at rates sufficient to support active shoot growth, but not tissue proliferation or lateral bud growth. Sometimes gibberellic acid is advantageously added to the medium to make shoots longer and thus facilitate single node separation.

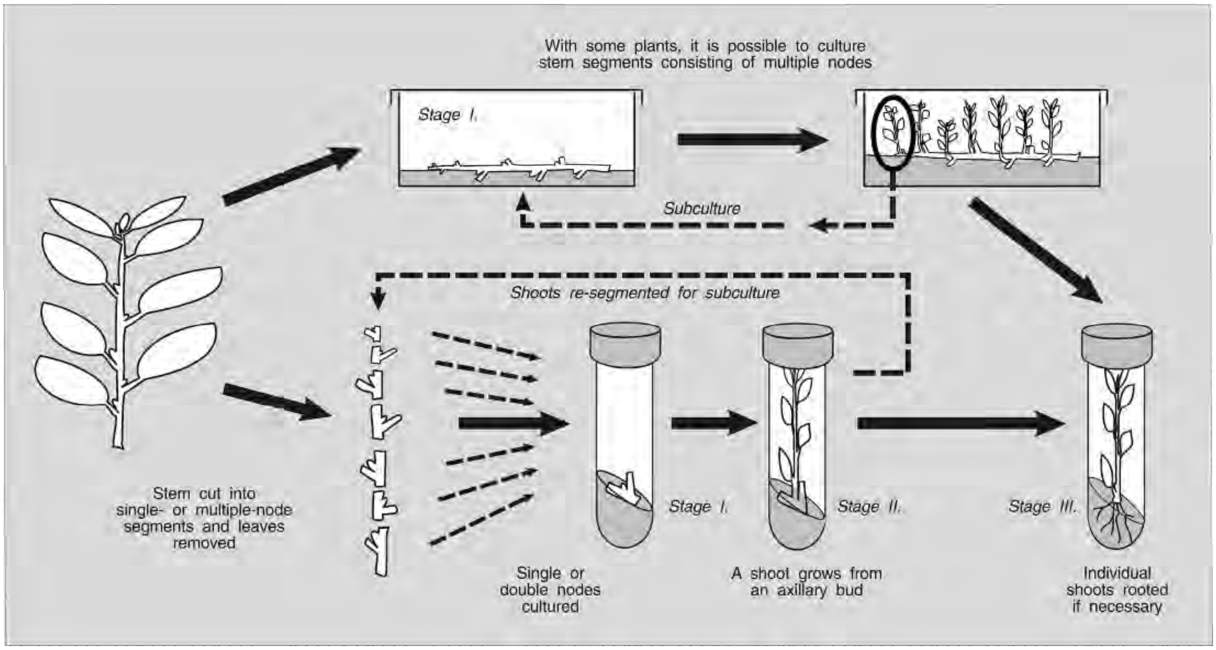

Fig. 2.4 Single and multiple node culture
Stage one cultures can be initiated also from meristem or shoot tips.

Current applications

Node culture is of value for propagating species that produce elongated shoots in culture (e.g. potato and *Alstroemeria*), especially if stimulation of lateral bud break is difficult to bring about with available cytokinins. Nowadays the technique becomes more and more popular in commercial micropropagation. The main reason is that it gives more guarantee for clonal stability. Indeed, although the rate of multiplication is generally less than that which can be brought about through shoot culture, there is less likelihood of associated callus development and the formation of adventitious shoots, so that Stage II subculture carries very little risk of induced genetic irregularity. For this reason, node culture has been increasingly recommended by research workers as the micropropagation method least likely to induces somaclonal variation.. Some of the plants for which node culture has been described are listed in Table 2.2.

3.1.4. Multiple shoots from seeds (MSS)

During the early 1980's it was discovered that it was possible to initiate multiple shoot cultures directly from seeds. Seeds are sterilised and then placed onto a basal medium containing a cytokinin. As germination occurs, clusters of axillary and/or adventitious shoots ('multiple shoots') grow out, and may be split up and serially subcultured on the same medium. High rates of shoot multiplication are possible. For instance, Hisajima (1982a) estimated that 10 million shoots of almond could be derived theoretically from one seed in a year.

It is likely that multiple shoots can be initiated from the seeds of many species, particularly dicotyledons. The technique is effective in both herbaceous and woody species: soybean (Cheng *et al.,* 1980; Hisajima, 1981; Hisajima and Church, 1981): sugar beet (Powling and Hussey, 1981): almond (Hisajima, 1981; 1982a,b,c): walnut (Rodriguez, 1982): pumpkin and melon (Hisajima, 1981): cucumber and pumpkin (Hisajima, 1981, 1982c): pea, peanut, mung bean, radish, *Zea mays* and rice (Hisajima, 1982c). This technique does only make sense when elite seed is used or to gain preliminary information on the behaviour of a plant species under *in vitro* conditions.

Table 2.2 Examples of the use of node culture in micropropagation

Monocotyledons		*Solanum* spp.	Haberlach *et al.* (1985), Levy (1988), Sihachakr *et al.* (1988)
Alstroemeria	Hussey *et al.* (1980)	**Woody dicotyledons**	
Cymbopogon spp.	Jagadish Chandra and Sreenath (1982)	*Carpinus betulus*	Chalupa (1981a)
Poa pratensis	Pieper and Smith (1988)	*Castanea sativa* *Castanea mollissima*	Vieitez and Vieitez (1980b) Yang *et al.* (1986)
Asparagus officinalis	Yang and Clore (1973, 1974a)	*Eucalyptus grandis*	Cresswell and Nitsch (1975)
Dioscorea spp.	Ammirato (1976, 1982), Chaturvedi and sinha (1979b)	*Forsythia ovata*	Einset and Alexander (1985)
Zea mays	King and Shimamoto (1984)	*Fraxinus pennsylvanica*	Einset and Alexander (1985)
Orchid monocots		*Jugans regia*	Dandekar *et al.* (*1988)*
Dendrobium spp.	Ball and Arditti (1976)	*Hevea brasiliensis*	Chen Z. (1984)
Phalaenopsis spp.	Tanaka and Sakanishi (1978)	*Leucaena obtusifoliun*	Einset and Alexander (1985)
Thunia alba	Singh and Prakash (1984)	*Lonicera periclymemum*	Boonnour *et al.* (1988)
Vanilla planifolia	Kononowicz and Janick (1984a)	*Olea europea*	Rugini and Fontenazza (1981)
Herbaceous dicotyledons		*Paulownia tomentosa*	Burger *et al.* (1985)
Angelonia salicariaefolia	Datta and Datta (1984)	*Poncirus trifoliate*	Barlass and Skene (1982b)
Cucumis sativus	Handley and Chambliss (1979)	*Prosopis julflora*	Wainwright and England (1987)
Glycyrrhiza glabra	Shah and Dalal (1980, 1982)	*Prunus armeniaca*	Snir (1984)
Rosmarinus officinalis	Misra and Chaturvedi (1984)	*Quercus robur*	Chalupa (1984a,b)
Rorippa nasturtium	Wainwright and Marsh (1986)	*Salix* spp.	Chalupa (1981a, 1983)
Solanum tuberosum	Hussey and Stacey (1981a) Kristensen (1984)	*Syringa* spp.	Einset and Alexander (1985)
		Syringa x *chinensis*	Welander N.T. (1987)

3.1.5. Shoots from floral meristems

Meristems that would normally produce flowers or floral parts can sometimes be induced to give vegetative shoots *in vitro*. Success depends on the use of young inflorescences where the determination of individual flower meristems is not canalized. Meristems in older inflorescences are likely to give rise to floral structures. Culture of immature inflorescence segments has, for example, resulted in shoot formation in:

- Bamboo - Gielis & Debergh (1998)
- Broccoli - Anderson and Carstens (1977)
- Cauliflower - Pow (1969), Margara (1969a,b,c; 1977a), Crisp and Walkey (1974), Grout and Crisp (1977), Trimboli et al. (1977)
- Coconut - Eeuwens and Blake (1977)
- *Dendranthema* - Shu O Wang and Su Shien Ma (1978)
- Date palm - Drira and Benbadis (1985)
- *Gerbera* - Topoonyanont and Dillen (1988)
- *Limonium* - Topoonyannt *et al*. (1999)
- Onion - Dunstan and Short (1977b; 1979a)
- Sugar beet - Coumans-Gilles *et a*l. (1981)

The exact origin of the shoots produced has not always been determined. In cauliflower and coconut they were thought to originate from actual flower meristems, but in sugar beet, from floral axillary buds. Some shoots formed from onion flower heads arose from various parts of the flower buds, but they were accompanied by other shoots which arose adventitiously over the entire receptacle surface. Shoots formed from young flower buds may therefore

not always result from the reversion of floral meristems. In fact the direct formation of adventitious shoots is more widely reported.

3.2. PROPAGATION BY DIRECT ORGANOGENESIS

3.2.1. Direct adventitious shoot initiation

In certain species, adventitious shoots which arise directly from the tissues of the explant (and not within previously-formed callus) can provide a reliable method for micropropagation. However, the induction of direct shoot regeneration depends on the nature of the plant organ from which the explant was derived, and is highly dependent on plant genotype. In responsive plants, adventitious shoots can be formed *in vitro* on pieces of tissue derived from various organs (e.g. leaves, stems, flower petals or roots); in others species, they occur on only a limited range of tissues such as bulb scales, seed embryos or seedling tissues. Direct morphogenesis is observed rarely, or is unknown, in many plant genera.

Direct shoot formation is sometimes accompanied by proliferation of unorganised cells, and a regenerative tissue that could be classed as callus, may ultimately appear. Its formation can usually be reduced by adjustment of the growth regulators in the medium. Because there is a risk of regenerating plants with a different genetic identity (see Vol. 2), use of the callus for further propagation is not recommended unless it has a highly organised nature (see later). In some instances, the growth regulators used to initiate shoot buds directly on explants may not be conducive to continued bud growth. A closely packed mass of shoot primordia may then be mistaken for organised callus.

In those species where adult tissues have a high regenerative capacity, the main advantages of micropropagation by direct adventitious shoot regeneration are that:

- Initiation of Stage I cultures and Stage II shoot multiplication, are more easily achieved than by shoot culture. It is, for example, simpler to transfer aseptically several pieces of *Saintpaulia* leaf petiole to culture medium, than to isolate an equivalent number of shoot meristems.
- Rates of propagation can be high, particularly if numerous small shoots arise rapidly from each explant.

Stage I

Stage I consists of the establishment *in vitro* of suitable pieces of tissue, free from obvious contamination. As adventitious shoots are usually initiated on the tissue without transfer, Stages I and II are not generally discrete.

Stage II

Initially Stage II of this micropropagation method is recognised by the formation, growth and proliferation of adventitious shoots from the primary explant. Subsequently Stage II subcultures might, theoretically, be established from individual shoots by the techniques familiar in shoot culture. In practice, in plants such as *Saintpaulia*, both further adventitious and axillary shoots may develop in later stages of propagation. The result is a highly proliferative shoot mass and a very rapid rate of propagation. Subcultures are made by transferring shoot clumps (avoiding basal callus) to fresh media. In most commercial laboratories the micro-propagation of *Anthurium* species is initiated by adventitious shoot formation on leaf explants, followed by only axillary shoot development during the succeeding subcultures (Debergh *et al*,, 1990). Adventitious shoots sometimes arise directly from the leaves of plants during shoot culture. This often happens when leaves bend down to touch the semi-solid medium. Adventitious shoot formation of certain plants will take place in large vessels of aerated liquid medium, allowing the scale of propagation to be much increased (see the discussion on liquid media in Chapter 12).

Stage III

This is similar to the Stage III of most propagation systems. Individual shoots or shoot clumps are transferred to a nutrient medium with added growth regulators and ingredients that do not encourage further shoot proliferation and which promote rooting; alternatively shoots may be removed from culture and rooted *ex vitro*.

Some current applications

Several ornamental plants are at present propagated *in vitro* by direct shoot regeneration. Chief among these are plants of the family Gesneriaceae, (including *Achimenes, Saintpaulia, Sinningia* and *Streptocarpus*), where shoot buds can be freely regenerated directly on leaf explants without the formation of any intervening callus phase. Many other ornamentals and crop plants either are (or could be) propagated efficiently by this means, for example, begonias, *Epiphyllum*, cacti, *Gerbera, Hosta* and *Lilium*. Further examples are quoted in the tables of Volume 2. Remember that this technique is more prone to yield off-types than shoot and node cultures,

and that the technology is not applicable for the propagation of chimeras.

Regeneration from root pieces

In vitro shoot regeneration from root pieces is mainly reported from plants that possess thick fleshy roots such as those of the genera *Cichorium, Armoracia, Convolvulus*, and *Taraxacum*. It is, however, a method of propagation that is potentially applicable to a wide range of species (Browse, 1980; Hodge, 1986). Shoots have, for instance, been induced to form directly on segments and apices of the roots of *Citrus* and *Poncirus* seedlings (Sauton *et al.,* 1982). Shoot regeneration from root pieces does not offer a continuous method of micropropagation unless there is a ready supply of aseptic root material (e.g. from isolated root cultures). Roots grown in soil *in vivo* are usually heavily contaminated and can be difficult to sterilize to provide an adequate number of uncontaminated cultures. They can however be used as an initial source of shoots which can be multiplied afterwards by shoot culture [e.g. *Robinia* (Chalupa, 1992)].

Tissue maceration or fragmentation

The capacity of young fern tissue to regenerate adventitious shoots can be very high. Fern prothallus tissue (the gametophyte generation produced from germinating spores) has a high capacity for regeneration; a new prothallus can usually be grown from small isolated pieces of tissue (Whittier and Steeves, 1962), or even from single cells produced by maceration (Miller J.H., 1968; De Fossard, 1976; Knauss, 1976). Plants can also be regenerated from homogenised sporophyte tissue of some fern genera, and homogenisation has been incorporated into tissue culture, or partial tissue culture techniques for the propagation of plants of this class (see Volume 2).

Because a high proportion of the direct cost of micropropagation is attributable to the manual separation and transfer of explants and cultured material between media, the ability to regenerate plants from macerated or fragmented tissue would be extremely advantageous. Unfortunately there seem to be only a limited number of publications describing the formation of shoots directly from machine-macerated tissue of higher plants. One of them is the patent of Lindemann (1984), the claims of which may have been somewhat optimistic. Also Levin *et al.* (1997) reported on the regeneration of different plant species using a homogenisation technology. However, shoot regeneration from fragmented shoot tips, or micropropagation of some plants by culturing shoot material or tissue fragments in fermentors (Vol. 2), are somewhat comparable.

3.2.2. Organised calluses

In most callus cultures, shoots are produced from meristems which arise irregularly and may therefore be genetically altered. By contrast, so-called ‘organised’ or ‘semi-organised’ calluses are occasionally isolated in which there is a superficial layer of proliferating shoot meristems, overlaying an inner core of vacuolated cells acting as a mechanical and nutritional support. Calluses of this kind were termed organoid colonies by Hunault (1979): the names *meristemoids* and *nodules* have also been proposed. A meristemoid is defined as a cluster of isodiametric cells within a meristem or cultured tissue, with the potential for developmental (totipotential) growth. Meristemoids may give rise to plant organs (shoots, roots) or entire plants in culture (Donnelly and Vidaver, 1988). Nodules also comprise meristematic cells, but they are distinct from meristemoids because they are independent spherical, dense cell clusters which form cohesive units, with analogy to both mineral nodules in geology and root nodules of legumes (McCown *et al.,* 1988). Nodule culture has been extensively used for the propagation of *Cichorium intybus* (Pieron *et al.,* 1993).

The presence, in meristemoids, of an outer layer of shoot meristems seems to inhibit the unbridled proliferation of the unorganised central tissue (Hussey, 1983). Geier (1988) has suggested that the control mechanisms which ensure the genetic stability of shoot meristems are still fully, or partly, active. Maintenance of a semi-organised tissue system depends on a suitable method of subculture and upon the use of growth regulator levels which do not promote excessive unorganised cell growth. Repeated selective transfer of unorganised portions of an organised *Anthurium scherzerianum* callus eventually resulted in the loss of caulogenesis (Geier, 1986). Conversely, by consistently removing the unorganised tissue when subculturing took place, shoot formation from the callus was increased.

Cultures consisting of superficial shoot meristems above a basal callus, seem to occur with high frequency amongst those initiated from meristem tip, or shoot tip, explants. Hackett and Anderson (1967) induced the formation of tissue of this type from carnation shoot tips by mutilating them with a razor blade before culture. Similar cultures were also obtained from seedling plumular tip explants of two (out of five tested) varieties of *Pisum sativum* placed

on an agar medium (Hussey and Gunn, 1983; 1984). The calli were highly regenerative for 2-3 years by regular subculture to agar or shaken liquid medium. Maintenance was best achieved with an inoculum prepared by removing larger shoots and chopping the remainder of the callus and small shoots into a slurry. A callus, formed at the base of *Solanum curtilobum* meristem tips on filter paper bridges, gave rise to multiple adventitious shoots from its surface when transferred to shake culture in a liquid medium (Grout *et al.,* 1977).

Callus with superficial proliferative meristems has also been induced by culture of shoot or meristem tips on a rotated liquid medium, in:

- *Nicotiana rustica* (Walkey and Woolfitt, 1968);
- *Chrysanthemum morifolium* (Earle and Langhans 1974c);
- *Stevia rebaudiana* (Miyagawa *et al.,* 1986).

In *Stevia rebaudiana* (above), a slow rotation speed (2 rpm) was essential for initiation of an organised callus. A small callus formed upon the explant and in 2-3 weeks came to possess primary superficial shoot primordia which were globular and light green. Dark green aggregates of shoot primordia (termed 'secondary shoot primordia'' by Miyagawa *et al.,* 1986) were developed within 6 weeks. If divided, the aggregations of shoot initials in both *Nicotiana* and *Stevia* could be increased by subculture or, if treated to a different cultural regime, could be made to develop into shoots with roots. Shoots were produced from *Chrysanthemum* callus upon subculture to an agar medium. A spherical green dome-like structure was produced from meristem tips dissected from germinated *Eleusine coracana* (Gramineae) caryopses. When cut into four and subcultured, a green nodular structure was formed which grew to 5-10 mm in diameter. It was similar in appearance to a shoot dome, but much larger (a natural shoot dome is only 70-80 μm wide). The nodular structures were termed 'supradomes' by Wazizuka and Yamaguchi (1987) because, unlike normal callus, superficial cells were arranged in an anticlinal plane and those beneath had a periclinal arrangement. Numerous multiple buds could be induced to form when the organised tissue was subcultured to a less complex medium.

Although proliferative meristematic tissue formed from shoot tips always appears to be accompanied by a basal callus, the superficial meristematic cells may well be derived directly from the cells of the apical shoot meristem of the explant, for they preserve the same commitment to immediate shoot formation. The presence of the apical meristem in the explant seems to be essential and culture of tissue immediately beneath it does not produce a callus with the same characteristics (Hussey and Gunn, 1984). Similar semi-organised callus can appear at the base of conventional shoot cultures. In the green granular callus mass which formed at the base of *Rhododendron* shoot tips, each granule represented a potential shoot (Kyte and Briggs, 1979). Organised caulogenic callus is thus closely comparable to embryogenic callus formed from pre-embryogenically determined cells.

Organised callus can be produced from explants other than shoot tips; in *Anthurium*, it has been derived from young leaf tissue (above, Geier, 1986) and from spadix pieces (Geier, 1987).Organised callus has two characteristics which distinguish it from normal unorganised callus: the plants produced from it show very little genetic variation, and it can be subcultured for a very long period without losing its regenerative capacity. The callus of *Nicotiana* (above) was able to produce plantlets over a ten-year period, while that of *Chrysanthemum* gave rise to plants continuously during four years.

The use of cultures with superficial proliferative meristems has not yet been widely used for micropropagation. There are three possible reasons:

- the genetic variation which is almost invariably induced by shoot regeneration from normal callus, has cautioned against the use of any sort of callus culture for this purpose;
- organised callus may not always be readily distinguished from its unorganised counterpart;
- methods of initiating organised callus in a predictable fashion have not yet been fully elucidated.

There are examples of the initiation of organised callus from a sufficiently wide range of plant species (particularly from meristem tip explants) to suggest that it could be a method of general applicability. Multiplication may well be amenable to large-scale culture in fermentors (Vol. 2).

3.2.3 Direct embryogenesis

Somatic embryos are often initiated directly upon explanted tissues. Of the occurrences mentioned in Chapter 1, one of the most common is during the *in vitro* culture of explants associated with, or immediately derived from, the female gametophyte. The tendency for these tissues to give rise to adventitious somatic embryos is especially high in plants where sporophytic polyembryony occurs

naturally, for example, some varieties of *Citrus* and other closely related genera.

Ovules, nucellar embryos, nucellus tissues and other somatic embryos are particularly liable to display direct embryogenesis. In *Carica* somatic embryos originated from the inner integument of ovules (Litz and Conover, 1981a,b) and in carrot tissue of the mericarp seed coat can give rise to somatic embryos directly (Smith and Krikorian, 1988).

The nucellus tissue of many plants has the capacity for direct embryogenesis *in vitro* (Haccius and Hausner, 1976; Eichholtz *et al.,* 1979; Rangaswamy, 1982; Litz, 1987). As explained in Chapter 1, explants may also give rise to a proliferative tissue capable of embryogenesis. The high embryogenic competence of the nucellus is usually retained during subsequent cell generations *in vitro*, should the tissue be induced to form 'callus' (or cell suspensions). It is not clear whether all cells of the nucellus are embryogenically committed. In *Citrus,* somatic embryos are formed from the nucellus even in cultivars that are normally monoembryonic (i.e. the seeds contain just one embryo derived from the zygote), whether the ovules have been fertilised or not. It has been suggested that only those cells destined to become zygotic proembryos can become somatic proembryos or give rise to embryogenic callus (Sabharwal, 1963); somatic embryos have been shown to arise particularly from the micropylar end of *Citrus* nucellus.

Adventitious (adventive) embryos are commonly formed *in vitro* directly upon the zygotic embryos of monocotyledons, dicotyledons and gymnosperms, upon parts of young seedlings (especially hypocotyls and cotyledons) and upon somatic embryos at various stages of development (especially if their growth has been arrested). The stage of growth at which zygotic embryos may undergo adventive embryogenesis is species-dependent: in many plants it is only immature zygotic embryos which have this capacity. Unfortunately, as the phenotypic potential of seedlings is rarely known, using them as a source of clonal material is of limited value.

Embryogenic determination can be retained through a phase of protoplast culture. Protoplasts isolated from embryogenic suspensions, may give rise to somatic embryos directly, without any intervening callus phase (Miura and Tabata, 1986; Sim *et al.,* 1988). Treating protoplasts derived from leaf tissue of *Medicago sativa* with an electric field, induced them to produce somatic embryos directly upon culture (Dijak *et al.,* 1986).

Adventitious embryos arising on seedlings are sometimes produced from single epidermal cells (Konar *et al.,* 1972a; Thomas *et al.,* 1976 - see Chapter 1). Zee and Wu (1979) described the formation of proembryoids within petiole tissue of Chinese celery seedlings, and Zee *et al.* (1979) showed that they arose from cortical cells adjacent to the vascular bundles which first became meristematic. Hypocotyl explants from seedlings of the leguminous tree *Albizia lebbek* showed signs of cracking after two weeks of culture and frequently young embryoids emerged (Gharyal and Maheshwari, 1981). Stamp and Henshaw (1982) found that primary and secondary embryogenesis occurred in morphogenically active ridges produced on the surface of cotyledon pieces taken from mature cassava seeds.

Somatic embryos have been observed on the roots and shoots of *Hosta* cultures (Zilis and Zwagerman, 1980) and on the needles and cultured shoots of various gymnosperm trees (Bonga, 1976; McCown and Amos, 1982).

Protocorm formation in orchids

The seeds of orchids (like those of some other saprophytic or semi-parasitic plants) contain a small embryo of only about 0.1 mm diameter, without any associated endosperm storage tissue. Upon germination, the embryo enlarges to form a small, corm-like structure, called a protocorm, which possesses a quiescent shoot and root meristem at opposite poles. In nature, a protocorm becomes green and accumulates carbohydrate reserves through photosynthesis. Only when it has grown and has sufficient stored organic matter does it give rise to a shoot and a root. Normal seedling growth then continues utilising the stored protocorm food reserves.

Bodies which, in their structure and growth into plantlets, appear to be identical with seedling protocorms (except that on synthetic media they may not be green), are formed during *in vitro* culture of different types of orchid organs and tissues. These somatic protocorms can appear to be dissimilar to seedling protocorms, and many workers on orchid propagation, have used terms such as 'protocorm-like bodies' (PLBs) to describe them.

When a shoot tip of an orchid is transferred to culture on a suitable medium, it ceases to grow and to develop as a mature shoot apex; instead it behaves as though it were the apex of an embryo, i.e. it gives rise

to a protocorm (Vol. 2). Protocorm-like bodies also arise directly on some other orchid explants and proliferate from other PLBs in a fashion which is exactly comparable to the direct formation of somatic embryos.

Champagnat and Morel (1972) and Norstog (1979) considered the appearance of protocorms to be a manifestation of embryogenesis because they represent a specialised stage in embryo development and are normally derived directly from zygotic embryos. We think that this is the correct interpretation: in a previous edition of this book, protocorms were described under 'storage organs'.

Other protocorm-like structures. *In vitro* culture of small immature proembryos from developing barley seeds (Norstog, 1961, 1965a, 1970) or from the fern *Todea barbara* (De Maggio and Wetmore, 1961) has been noted to result in the formation of protocorm-like tissue masses from which root and shoots are regenerated after a period of irregular growth. Mapes (1973) recorded the appearance of such protocorm-like structures on shoot tips of pineapple, and Abo El-Nil and Zettler (1976) describe their direct formation on shoot tip explants of the yam *Colocasia esculenta*, or indirectly in subsequent callus cultures.

Embryogenesis from microspores or anther culture

Somatic embryos can be initiated directly from microspores. Usually it is necessary to culture the microspores within anthers, but occasionally it has been possible to induce embryogenesis from isolated microspores. Anther and microspore culture are described in Chapter 1, but because the plants produced by anther culture are likely to be dissimilar to their parents, we shall not consider the method in any detail in this book, and reports of anther culture have been largely omitted from the tables in Volume 2. Good references to this topic are available in the series of books published by Jain *et al.* (1996 and 1997).

Anther culture can result in callus formation; the callus may then give rise to plants through indirect embryogenesis or adventitious shoot formation.

Embryo proliferation

Accessory embryos on zygotic embryos. Occasionally new somatic embryos are formed directly on zygotic embryos that have been transferred to *in vitro* culture. Such adventitious embryos have been reported, for example, in: *Cuscuta reflexa* (Maheshwari and Baldev, 1961); barley (Norstog, 1970); *Ilex aquifolium* (Hu and Sussex, 1972; Hu, 1977; Hu *et al.,* 1978); *Thuja orientalis* (Konar and Oberoi, 1965); *Trifolium repens* (Maheswaran and Williams, 1985); *Zamia integrifolia* (Norstog, 1965b; Norstog and Rhamstine, 1967); *Theobroma cacao* (Pence *et al.,* 1980a,b); *Linum usitatissimum* (Pretova and Williams, 1986); *Vitis vinifera* (Stamp and Meredith (1988).

When direct embryogenesis occurs on pre-formed embryonic tissue, the newly formed embryos are sometimes termed direct secondary embryos or accessory embryos.

Accessory embryos on somatic embryos The *in vitro* induction of somatic embryogenesis starts a highly repetitive process, lacking some of the controls which must exist in nature during the formation of zygotic embryos. This results in the frequent development of small additional embryos on somatic embryos which have arisen directly on explants, or indirectly in callus and suspension cultures. Accessory embryos can occur along the whole axis of the original embryo, or grow preferentially from certain sites (e.g. the hypocotyl region or the scutellum of monocot. embryoids). In walnut, accessory embryos appear to arise from single cells of the epidermis of somatic embryos (McGranahan *et al.,* 1988).

Sometimes the term polyembryony is used to describe the formation of accessory, or secondary, embryos (Radojevic, 1988) (c.f. the term polyembryogenesis in Chapter 1). The process has also been called repetitive embryogenesis (Tulecke and McGranahan, 1985) or recurrent somatic embryogenesis (Lupotto, 1986). Such additional embryos are liable to be developed during all kinds of *in vitro* embryogenesis. They have been noted for example, on the somatic embryos formed in:

- Anther cultures: *Atropa belladonna* (Rashid and Street, 1973); *Brassica napus* (Thomas *et al.,* 1976); *Carica papaya* (Tsay and Su, 1985); *Citrus aurantifolia* (Chaturvedi and Sharma, 1985); *Datura innoxia* (Geier and Kohlenbach, 1973); *Vitis hybrids* (Rajasekaran and Mullins, 1979);
- Suspension cultures: *Daucus carota* (Ammirato and Steward, 1971; McWilliam *et al.,* 1974); *Ranunculus scleratus* (Konar and Nataraja, 1965b; Konar *et al.,* 1972a);
- Callus cultures: *Aesculus hippocastanum* (Radojevic, 1988); alfalfa (when individual embryoids were transferred to a fresh medium) (Saunders and Bingham, 1972); carrot (Petrù, 1970); Citrus (Button and Kochba, 1977); parsley (Vasil and

Hildebrandt, 1966b); *Pennisetum purpureum* (Wang and Vasil, 1982); *Ranunculus sceleratus* (Konar and Nataraja, 1965a,b), *Theobroma cacao* (Li *et al.,* 1998).

Protocorms arising directly on explanted shoot tips or leaf pieces of orchids, frequently produce other adventive 'daughter' protocorms in culture, in a fashion that is similar to the adventive formation of somatic embryos. Somatic embryos formed in callus of oil palm have been reported to give rise to protocorm-like bodies, which regenerated shoots repeatedly as subculture was continued (Paranjothy and Rohani, 1982).

Practical uses in propagation

From a quantitative point of view, indirect embryogenesis does provide an efficient method of micropropagation; the same is not true of direct embryogenesis when it is unaccompanied by the proliferation of embryogenic tissue. Although plants can be regenerated from embryos directly initiated *in vitro*, and may be present in sufficient numbers for limited plant production in breeding programmes, the numbers of primary embryos per explant will usually be inadequate for large scale cloning. To increase the number of somatic embryos formed directly on immature zygotic embryos of sunflower, Freyssinet and Freyssinet (1988) cut larger zygotic embryos into four equal pieces.

Additional embryos are generally unwanted: they are frequently joined one to another as twins or larger groups so that abnormal seedlings with multiple shoots develop from them. The presence of accessory embryos can also impede the growth of the primary somatic embryo. Growth then becomes asynchronous and normal seedlings may not be obtained unless the adventive embryos are removed.

However it has been suggested that accessory embryos might be used for micropropagating some species. Perhaps this is a method of micropropagation which will be developed more in the future? Examples of where it has been successful are:

- *Helianthus annuus* (Plissier *et al*.. 1990);
- *Juglans regia*. (McGranahan *et al*.1988b);
- *Medicago sativa* (Lupotto 1986).

As mentioned earlier embryogenesis has a great potential for mass propagation, however, all adventitious techniques do still have the associated problem of the lack of clonal stability. Therefore the commercial application of this technology remains limited except, perhaps, where embryos arise directly from parental tissue.

The propagation of orchids

Morel (1960) noticed that when protocorms of *Cymbidium* were divided new protocorms were formed from the pieces, whereas if they were not divided, original and regenerated protocorms developed into new plantlets. Morel (1960, 1964) suggested that meristem or shoot tip explants could be used to establish cultures for the clonal propagation of orchids, providing thereby the basis of the method which is now used for many orchid genera. Rates of propagation are improved through the use of slightly more complex media than used by Morel, and by including growth regulators. However, many commercial micropropagation laboratories do not favour the use of protocorms for micropropagation because of the lack of clonal fidelity.

Some orchids not only form protocorms on apical meristems, but also directly on explants such as leaves (Churchill *et al.,* 1971; 1973; Tanaka *et al.,* 1975), or flower stalks (Flamée and Boesman, 1977; Arditti *et al.,* 1977), or they may be formed from callus or callus via suspension cultures (see the section on orchids in Volume 2).

3.3. PROPAGATION BY INDIRECT ORGANOGENESIS

Propagation by all methods of indirect organogenesis carries a risk that the regenerated plants will differ genetically from each other and from the stock plant. Propagation by indirect organogenesis is described here for the sake of completeness; because of its potential as a propagation method, if the occurrence of genetic variation can be controlled; and because it is necessary for the regeneration and propagation of plants, which have been genetically transformed.

3.3.1. Indirect adventitious shoots from callus

Because they are not formed on tissues of the original mother plant, shoots (or other organs) are said to be regenerated indirectly when they are formed on previously unorganised callus, or in cell cultures. Separate root and shoot initials are characteristically formed in callus cultures and are only observed occasionally in suspensions where they are typically produced in large cell aggregates. Somatic embryogenesis occurs in both callus and suspension cultures. Adjustment of the growth regulators in the culture medium can bring about shoot or root formation in callus from a very large number of species. Inception of roots and shoots is most frequent in tissues that have been recently

isolated, and morphogenic capacity generally declines with time as the tissues are subcultured. Nevertheless, some callus cultures maintain their regenerative ability over long periods.

As explained in Chapter 10, callus cultures vary in their morphogenic potential or competence. Because of this, the callus which originates from some plants, or from some kinds of explant, may not be responsive to techniques and media which frequently result in morphogenesis. The tissue may be non-morphogenic, or may only produce roots, from which plants cannot be regenerated. In some cases callus lines with different appearances (texture, colour, etc) and/or morphogenic capacities can be isolated from the same explant (Fig 2.5). These differences may reflect the epigenetic potential of the cells, or be caused by the appearance of genetic variability amongst the cells of the culture (Volume 2) and support Street's (1979) suggestion that primary explants may be composed of cells or tissues capable of morphogenesis (competent cells) and others that are incapable (non-competent cells). Another possibility is that the operator is not able to create the appropriate conditions to express the full potential of the plant material he is working with.

Morphogenic and non-morphogenic callus lines, selected from primary callus, can retain their characteristics over many years (Reuther, 1990). Special treatments, such as, a change of medium, an altered cultural environment, or an adjustment of the growth regulators added to the medium, may induce shoots or roots to form in some apparently non-morphogenic calluses; but generally, treatments to reverse a non-regenerative condition are unsuccessful.

In practice, the speed and efficiency with which plantlets can be regenerated from callus depends upon:

- the interval between culture initiation and the onset of morphogenesis;
- the choice of the appropriate type of callus;
- the frequency and rate of shoot bud initiation;
- whether shoot regeneration can be readily re-induced when the callus is subcultured;
- the number of subcultures that are possible without loss of morphogenesis;
- whether newly-initiated buds can be grown into shoots capable of being isolated and subsequently rooted.

Normal callus cultures produce shoots relatively slowly, but from some plants, and certain explants, under conditions that are not yet fully understood, callus can be initiated which has an especially high ability to regenerate shoots or somatic embryos.

Stage I.

In most herbaceous broad leafed plants, it is possible to initiate morphogenically competent callus cultures from explants derived from many different tissues. Leaf, stem or root segments, pieces of storage tissue (e.g. tubers), seed embryos, shoot tips and seedling tissues have been used at various times. In monocotyledons there is a narrower range of suitable organs; embryos, very young leaf tissue, stem nodes and immature inflorescences being the most common sources. Initiation of callus cultures of many tree species, including gymnosperms, is frequently limited to explants derived from tissues near the vascular bundles or the cambium of stem or root sections. Explants containing actively dividing cells may be necessary if callus possessing a high level of morphogenic competence is to be isolated.

Callus growth is usually initiated by placing the chosen explant on a semi-solid medium into which auxin has been incorporated at a relatively high level, with or without a cytokinin. Details of the compounds used are given in Chapter 5. One or more transfers on the same medium may be necessary before the callus is separated from the parental tissue for subculture. Because more than one kind of callus may arise from a single explant, successful propagation can depend on being able to recognise and subculture only the type (or types) which will eventually be able to give rise to shoots or somatic embryos. In the absence of previous experience, samples of each type of callus may have to be carried forward for testing on inductive media. Translucent, watery callus is seldom morphogenic, whereas nodular callus frequently is.

Organised adventitious shoots are usually induced to form in callus or suspension cultures by reducing the auxin level in the medium and/or increasing the concentration of cytokinin. To grow callus-derived shoots into plantlets capable of survival in the soil, they must be rooted as micro-cuttings. Root production by callus is of little consequence for micropropagation purposes; even if roots are formed concurrently with adventitious shoots, the vascular connections between roots and shoots, through the callus tissue, are almost invariably insufficient for the development of a functional plantlet.

Stage II.

Once a morphogenic callus has been isolated, propagation is carried out either by callus subdivision, or by the preparation of cell suspensions.

The success of each technique depends on the subcultured tissues or cells continuing to regenerate shoots.

Callus subdivision. Callus is cut into smaller pieces which increase in size when subcultured in a liquid or an agar-solidified medium. The callus can either be subdivided further, or shoot regeneration allowed to occur. This may take place on the same medium, or the callus may need to be transferred to another shoot-inducing medium. The organogenic capacity of callus is easily lost on repeated subculture. Use of high growth regulator levels can encourage the proliferation of non-regenerative callus which will displace tissues having the competence to form new shoots (e.g. in *Pelargonium*; Holdgate, 1977).

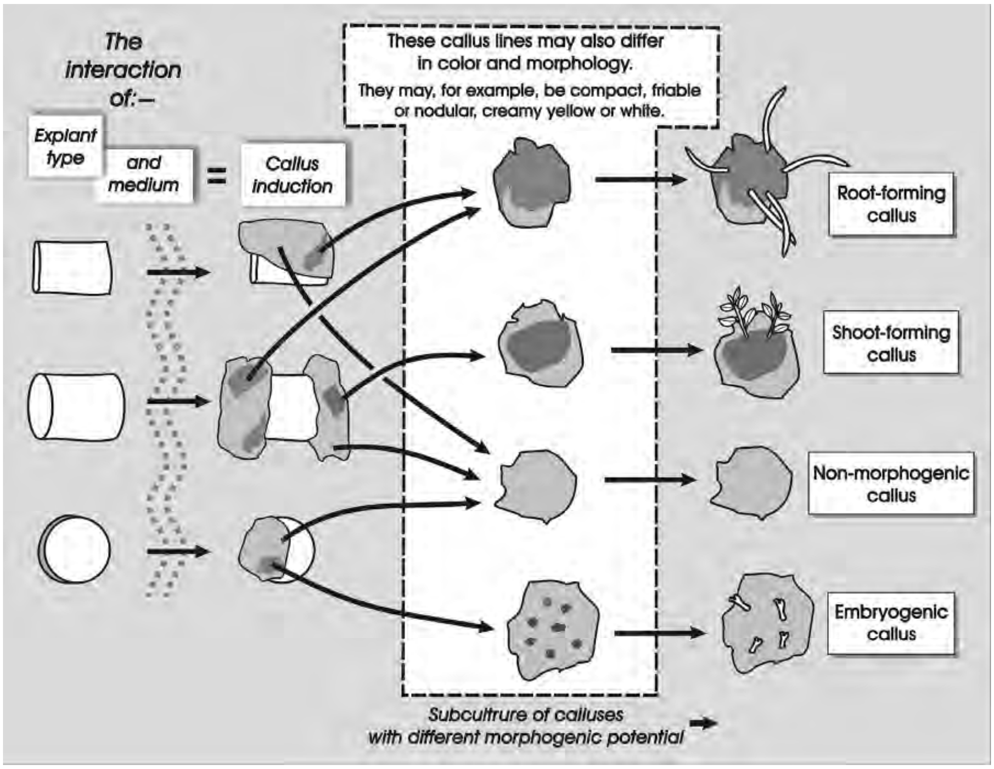

Fig. 2.5 Callus with different morphogenic potential is often isolated from a single explant.

The preparation of cell suspensions. Compared to the relatively rapid rates of propagation that are possible with shoot culture of some kinds of plants, propagation from morphogenically competent callus can be slow initially. Krikorian and Kann (1979) quoted a minimum of 135 days from the excision of daylily explants to the potting of plantlets. The rate at which propagation can proceed after that depends on the rate at which callus can be grown and subdivided. Providing a shoot-forming capacity is retained, a much faster rate of multiplication can be achieved by initiating a suspension culture from competent callus. After being increased by culture, the cells or cell aggregates can then be plated to produce new regenerative callus colonies. This is not an easy operation, as growth regulators favouring the formation of a dispersed cell suspension can cause the cells to lose their morphogenic capacity (see Chapter 10). There is also the problem that, by prolonging the period before shoot regeneration, genetic variability within the cell line will be increased.

Genetic stability

In some crop plants, the genetic differences between plants derived from callus and suspension cultures (discussed in Volume 2) are considerable, and are sufficient to have attracted the interest of plant breeders as a new source of selectable variability. However, plants obtained from callus lines with a high degree of morphogenic competence, appear to be much more uniform genetically. Care must be taken though to see that primary explants are

not taken from plant tissue likely to be endopolyploid. Subsequent exposure to high levels of growth substances such as 2,4-D should also be avoided as far as possible. Genetic stability of plants from highly competent callus cultures may be assisted by the continual presence of superficial meristems. As mentioned previously in Section 3.2.2, these probably repress shoot formation from cells within the callus mass (Hussey, 1983).

Morphogenic cereal cultures

The shoot forming capacity of some callus cultures has been attributed to the proliferation of meristematic centres derived from the tissues of the explant. King et al. (1978) have suggested that the small number of shoots produced by certain cereal tissue cultures arises in this way (e.g. in wheat, rice, oat and maize). Cure and Mott (1978) noticed that aberrant root-like structures existed within cereal cultures from which shoots arose. Such primordia, whether of root or shoot origin, are thought to proliferate adventitiously *in vitro*, surrounded by less organised tissues. Regenerative capacity is usually lost rapidly when the shoot primordia are diluted during subculture. Cereal callus of this kind does not have the same kind of inherent morphogenic capacity found in other types of callus cultures. Despite these observations, experience shows that morphogenesis can occur from previously unorganised cereal callus.

Current applications

In the past, several ornamental plants [e.g. *Freesia* (Hussey and Hargreaves, 1974) and *Pelargonium* (Holdgate, 1977)] have been micropropagated from adventitious shoots produced indirectly from callus. It had been hoped to extend the technique to other species possessing a strong natural tendency towards diploidy (e.g. some forest trees) where plantlets produced *in vitro* might have a normal karyotype (Mott, 1981), but it is now realised that the genetic changes which are almost universally induced in the genotype of cells during callus and cell culture make cloning by this technique inadvisable except where new genotypes are required for selection or further plant breeding. Another possibility is that mutated somatic cells, already present in the mother plant, are given the opportunity to develop into a plantlet.

3.3.2 Indirectly-initiated somatic embryos

Indirect formation of somatic embryos (or adventitious somatic embryogenesis) from callus or suspension cultures is observed more frequently than direct embryogenesis. Frequently callus which is wholly or partly embryogenic can be induced during the initial culture of explants derived from young meristematic tissues (see below), but induction is less common in cultures which have been kept and transferred for some period without organogenesis.

There are important requirements for the successful induction of embryogenic callus and suspension cultures:

- The plant genotype must be capable of embryogenesis on the chosen system of induction (medium plus added growth regulators). In some genera most genotypes are competent, but in others there may be a wide variation in competence even between different varieties or cultivars within a species.
- In most practical situations, cultures should be grown in the presence of an auxin for the induction (and initiation) of embryogenesis (Stage I).
- The level of sugar (e.g. sucrose or glucose) in the medium may need to be within critical concentrations, and no embryos may be formed at all if the sugar concentration is too high (Lippman and Lippmann, 1984).
- After the beginning of embryogenesis, it is usually (but not invariably) necessary for Stage I tissues or cells to be subcultured to a medium containing a reduced auxin concentration, or containing no auxin at all (Stage II) (Chapter 9).
- There may be an optimum length of time during which the Stage I routine should be maintained. An extended period before subculture can result in the failure to obtain embryogenesis at Stage II (e.g. Dos Santos *et al.*1980). Maintenance of the cultures on high auxin usually causes embryo development to be arrested or a loss of embryogenic capability.
- A supply of reduced nitrogen is required. This may be supplied in the form of NH_4^+ ion and/or as an amino acid such as glutamine or alanine (see Chapter 3).

Embryogenesis in primary callus cultures

Callus capable of producing somatic embryos (embryogenic callus) is most reliably obtained from an explant during the initial phase of culture, and is frequently produced in conjunction with non-morphogenic tissue. Embryogenic callus can usually be distinguished by its nodular appearance, and is frequently produced preferentially from one part of an explant (e.g. the scutellum of a monocotyledon embryo), probably because only the cells of that part of the explant were embryogenically pre-determined. These may be the same tissues, which in another

cultural environment are capable of producing embryos directly (Sharp *et al.,* 1980). According to this hypothesis, although competent and non-competent cells may produce callus, only that which grows from competent cells will give rise to somatic embryos.

The expression of competence depends on the use of a suitable medium for the culture, containing requisite growth regulators at the correct concentration. The formation of somatic embryos in *Lolium multiflorum*, for example, was medium dependent (Dale *et al.,* 1981). On the most suitable medium, immature inflorescence explants produced three types of callus, only one of which spontaneously formed embryo-like structures. Unless such different callus types are separated, cells of different regenerative capabilities may become mixed. Morphogenically competent cells could then be lost by competition in the combined callus tissue that results.

Stage I. Selection of an appropriate explant is most important. Embryogenic callus has been commonly gained from seed embryos, nucelli or other highly meristematic tissues such as parts of seedlings, the youngest parts of newly initiated leaves and inflorescence primordia. Within an inflorescence, staminoids [*Theobroma cacao* (Li *et al.,* 1998)] and filaments [*Aesculus hippocastanum* (Radojevic, 1995)] have been reported to be adequate sources of explants. The initiation of embryogenic callus from root tissue is rare but has been reported in some monocotyledons e.g. rice (Inoue and Maeda, 1982; Toshinari and Futsuhara, 1985); oil palm (Paranjothy and Rohani, 1982), Italian ryegrass (Jackson and Dale, 1988) and *Allium carinatum* (Havel and Novak, 1988). Callus is usually commenced on a semi-solid medium incorporating a relatively high level of an auxin; compounds commonly used for this purpose are described in Chapters 11 and 12. Only a few tissues with a high natural embryogenic capacity do not require the addition of endogenous auxin for the development of embryogenic callus. Occasionally, primary callus arising from an explant may show no morphogenic capacity, but can be induced to give rise to new embryogenic tissue during later (secondary) subcultures by transfer to an inductive medium. Ahee *et al.* (1981) have used this method to propagate oil palms. On the medium used, calluses arising on the veins of young leaf fragments had no morphogenic capability. However, when primary calluses were subcultured onto appropriate media (unspecified), some of them gave rise to tissue that was different in structure and form, and grew at a much faster rate. These 'fast-growing calluses' could be induced to produce structures resembling embryoids, and afterwards plantlets, upon further subculture to other media.

One highly embryogenic tissue that has been extensively studied is that of the nucellus of the polyembryonic 'Shamouti' orange (Spiegel-Roy and Kochba, 1980). Here it seems that cells at just one end of the embryo sac (the micropylar end) are embryogenically predetermined and retain this capacity in subsequent cell generations. On subculture, proliferation of the nucellus cells proceeds without the addition of growth regulators to the medium, and results in the formation of an habituated callus. A tissue is said to have become habituated when it will grow without a growth regulator, or some other organic substance which is normally necessary, being added to the medium (see Chapter 7). Addition of auxins to the growth medium is inhibitory to the growth of auxin-habituated 'Shamouti' orange tissue, which has been thought to be composed (at least initially) of numerous pro-embryos and not of undifferentiated cells (Button *et al.,* 1974). Embryogenic callus has also been obtained from the nucellus tissue of other plants, mainly tropical fruit species (Litz and Jaiswal, 1991).

Stage II. As a general rule, somatic embryos formed on a medium containing a relatively high concentration of an auxin, will only develop further if the callus culture is transferred to a second medium from which auxin has been omitted, another 'less active'' auxin has been substituted, or the level of the original auxin much reduced. This treatment is occasionally ineffective (Handley and Sink, 1985) and sometimes adding a cytokinin helps to ensure embryo growth. A further essential requirement is the need for a supply of reduced nitrogen in the form of an ammonium salt or amino acid. No change of nitrogen source is required if MS medium was used for Stage I, but if, for example, White's medium were used for Stage I, it would need to be supplemented with reduced nitrogen, or the culture transferred to MS.

Callus subculture. Once obtained, embryogenic callus can continue to give rise to somatic embryos during many subcultures over long periods. The continued production of somatic embryos in these circumstances depends either on the continued proliferation of pro-embryogenic nodules, and/or the *de novo* formation of embryogenic tissue from young somatic embryos during each subculture. Inocula for

subcultures must be carefully selected. In wheat, callus with continued embryogenesis was only re-initiated from inocula taken close to somatic embryos; tissue from the same culture which did not contain embryos was not embryogenic in the next passage (Chu *et al.,* 1987).

Sometimes the number of embryos produced per unit weight of callus rises during a few passages and then slowly falls, the capacity to form somatic embryos eventually being irrevocably lost. Callus derived from the nucellus of 'Shamouti' oranges increased in its capacity to form somatic embryos when subcultured at 10-15 week intervals, while transfer at 4-5 week intervals, reduced embryogenesis (Kochba and Button, 1974).

Somatic embryos can be formed relatively freely in callus tissue, but where they are to be used for large scale propagation, their numbers can often be increased more rapidly and conveniently by initiating an embryogenic suspension culture from the primary callus (see below).

Embryogenesis in suspension cultures

Cultures from embryogenic callus (Stage I). Suspension cultures can sometimes be initiated from embryogenic callus tissue, and the cells still retain the capacity to regenerate somatic embryos freely. Obtaining such cultures is not always a simple matter, for the auxin levels that are often used to promote cell dispersion may result in the loss of morphogenic capability. Embryogenic cell suspensions are most commonly initiated from embryogenic callus that is placed in liquid medium on a shaker. Vasil and Vasil (1981a,b) and Lu and Vasil (1981a,b) have reported producing cultures of this type from pearl millet and guinea grass respectively. Suspensions were initiated and subcultured in MS medium containing 1-2.5 mg/l 2.4-D and 2.5-5% coconut milk, and came to be composed of a mixture of embryogenic cells (small, highly cytoplasmic and often containing starch) and non-embryogenic cells (large and vacuolated). Embryoids were induced to develop into somatic seedlings when plated onto an agar medium without growth regulators, or with lower levels of auxin than used at the previous stage.

Cultures from non-embryogenic sources. Embryogenesis can be induced in cell suspensions of some plants when the cultures are produced from non-morphogenic callus and have been maintained without morphogenesis for one or more transfers. Induction occurs most readily in recently isolated suspensions and usually becomes much less probable with increasing culture age. Loss of regenerative ability is often associated with the appearance of some cells with abnormal chromosome numbers, but it can also be due to culture on an inappropriate medium.

Embryogenesis in suspension cultures seems to require media at Stage I and Stage II with similar compositions to those necessary for somatic embryo formation in callus cultures. Somatic embryos can be formed in suspension cultures in very large numbers. Reinert *et al.* (1971) demonstrated that the continued capacity of carrot cell suspensions to form embryos depended on an adequate supply of nitrogen. Embryogenesis ceased on a medium containing little nitrogen, but it was re-induced for several transfers after the culture was returned to a high-nitrogen medium.

In a few kinds of plants it is possible to induce embryogenesis in previously unorganised suspension cultures. Success is so far recorded only in members of the families Apiaceae (Umbelliferae), Cruciferae and Scrophulariaceae. This is not therefore a method of propagation which can be readily utilised. There is a greater chance of obtaining an embryogenic suspension culture from embryogenic callus.

Abnormal embryos and plantlets

Unfortunately embryogenesis in both callus and suspension cultures is seldom synchronous so that embryoids at different stages of development are usually present in a Stage II culture from the onset. This presents a major drawback for plant propagation which could otherwise be very rapid, especially from suspensions. A proportion of the seedlings developing from somatic embryos can also be atypical: abnormalities include the possession of multiple or malformed cotyledons, more than one shoot or root axis, and the presence of secondary adventive embryos. Embryos with three cotyledons have been observed to give rise to well-formed plantlets (Smith and Krikorian, 1990). Abnormal somatic embryos do however produce secondary embryos, which are usually of normal morphology.

Pretova and Williams (1986) suggested that embryo proliferation, or 'cleavage' (see earlier in chapter), and the formation of accessory cotyledons and root poles, to be homologous with the production of discrete complete embryoids. They suggested that production of accessory cotyledons and somatic embryos on the hypocotyl, and additional root poles near the base of existing embryos, may represent either a gradient along these organs in the early stages of determination, or, be caused by a factor affecting cell to cell co-ordination.

Differential filtering and sedimentation to separate embryos at different stages of development (Giuliano et al., 1983) can improve the uniformity of embryo populations in suspension cultures. More recently image analysis has been used to select embryos in specific developmental stages (Kurata, 1995). In addition cultures can be maintained on media containing high levels of sucrose (Ammirato and Steward, 1971), and/or low levels of abscisic acid (Ammirato, 1973; 1974). Both approaches, as well as the addition of imazalil to the culture medium (Werbrouck *et al.,* 2000), limit the number of abnormalities and give a higher degree of synchronisation. High levels of sucrose and abscisic acid induce reversible dormancy in somatic embryos and thus might be used to temporarily suspend growth should this be advantageous in a planned micropropagation programme.

Dormancy is however not always reversible. Indeed somatic embryos can remain dormant, and conversion to plantlets can be problematic. Different types of approaches can be used to overcome this problem, i.e. desiccation, supplementing the medium with osmotic agents [e.g. polyethylene glycol (PEG), mannitol] (Capuana and Debergh, 1997).

Genetic stability

Plants regenerated through somatic embryogenesis are usually morphologically and cytologically normal, but sometimes a proportion of aberrant plants is obtained. Genetically abnormal plants are more likely to occur where embryogenesis is initiated in callus or suspension cultures after a period of unorganised growth or when embryogenic cultures are maintained for several months (Orton, 1985).

A proportion of albino plants lacking chlorophyll is characteristically produced in anther culture of cereals and grasses (Sunderland and Dunwell, 1977) and during embryogenesis from other monocot explants. Dale *et al.* (1980) found that plants produced from embryogenic callus cultures of Italian ryegrass were more likely to be devoid of chlorophyll the longer the cultures were maintained. After one year, some cultures produced only albinos. Embryo-like structures (although still present on the surface of the callus) tended to be distorted.

Current applications

Few plant species are at present propagated on a large scale via embryogenesis *in vitro*. This method of morphogenesis does however offer advantages which suggest that it will be used increasingly for plant cloning in the future:

- In some monocotyledons (e.g. cereals, date palm and oil palm) it provides a method of micropropagation where shoot culture has not been successful (but note however that in some attempts to clone oil palms through embryogenesis, the resulting plants have been very variable);
- Providing embryogenic cell suspensions can be established, plantlets can theoretically be produced in large numbers and at much lower cost because plantlets do not have to be handled and subcultured individually;
- Somatic embryos probably provide the only way for tissue culture methods of plant propagation to be economically deployed on extensively planted field crops and forest trees.

Techniques for the conversion (germination) and field planting of somatic embryos are discussed in Volume 2.

4. STORAGE ORGAN FORMATION

Many ornamental and crop species are normally propagated, stored and planted in the form of vegetative storage organs. It is therefore not surprising that, where such organs are produced *in vitro*, they often provide a convenient means of micropropagation and/or genotype storage. Characteristic, though small, storage structures can be induced to form in cultures of several plant species, for example:

Bulbils	*Amaryllis*, hyacinth, lily, onion *Narcissus*
Cormlets	*Gladiolus*
Miniature tubers	Potato, yams.

Protocorm formation as a method of propagation has been considered under Direct Embryogenesis.

Methods of obtaining storage organs vary according to the kind of tissue being cultured. Some storage organs formed *in vitro* can be planted *ex vitro* directly into the soil.

4.1. THE PRODUCTION OF BULBILS AND CORMLETS

Species that naturally produce bulbs can be induced to form small bulbs (bulbils or bulblets) in culture. Bulbils can be produced from axillary buds, but frequently they are formed from adventitious buds developed on pieces of leaf, on inflorescence

stalks, or on ovaries, and particularly on detached pieces of bulb scale.

Both axillary and adventitious shoots and bulbils are formed on bulb scale pieces *in vitro*. Strong dominance of the main shoot apex often prevents the formation of axillary buds at the bases of bulb scales *in vivo*, but buds capable of giving rise to bulblets (or to shoots upon which bulbs will be formed later) are freely produced when bulb scales or bulb sections are cultured. In some species it is important to include part of the basal plate in the explant. Depending on the kind of bulb being cultured, explants for continued Stage II propagation may consist of scales taken from bulblets, swollen shoot bases, or bulblets which have been trimmed and split. Propagules for transfer from Stage III to the external environment can be plantlets, plantlets with a bulblet at the base, or dormant bulblets.

Instead of producing storage organs composed of swollen leaf bases (bulbs), some monocotyledons store food reserves in swollen stem bases (corms). Small corms (cormlets) of *Gladiolus* may be formed directly on explanted tissue or on callus in culture (Ziv *et al.,* 1970; Ziv and Halevy, 1972), or they are produced on rooted plantlets grown in culture jars until the leaves senesce. Cormlets formed *in vitro* can be planted in soil or used to start new *in vitro* cultures (Hussey, 1978a,b).

The production of plantlets from genera producing bulbs and corms is discussed more fully in Volume 2.

Miniature tubers

Under appropriate environmental conditions, plants that naturally produce tubers can be induced to produce miniature versions of these storage organs, in a medium containing high cytokinin levels. Tubers normally formed on underground stolons are produced *in vitro* in axillary positions along *in vitro* shoots (Fig 2.6). Two crops where miniature tubers have been utilised for propagation are potato and yams. Methods for inducing the *in vitro* tuberization of potatoes were first described by Lin *et al.* (1978, in Wang and Hu, 1980) and Hussey and Stacey (1981a,b), and since by several other workers. Potato tubers form best in darkness, but those of *Dioscorea* mainly appear at the base of stem node cuttings in the light (Ng, 1988).

Miniature tubers have the great advantage that they can be readily removed from culture flasks in a dormant condition and stored *ex vitro* without precautions against sepsis. When planted in soil they behave as normal tubers and produce plants from axillary shoots. If they are produced *in vitro* from virus-tested shoots, miniature tubers provide an ideal method of propagating and distributing virus-tested stock to growers.

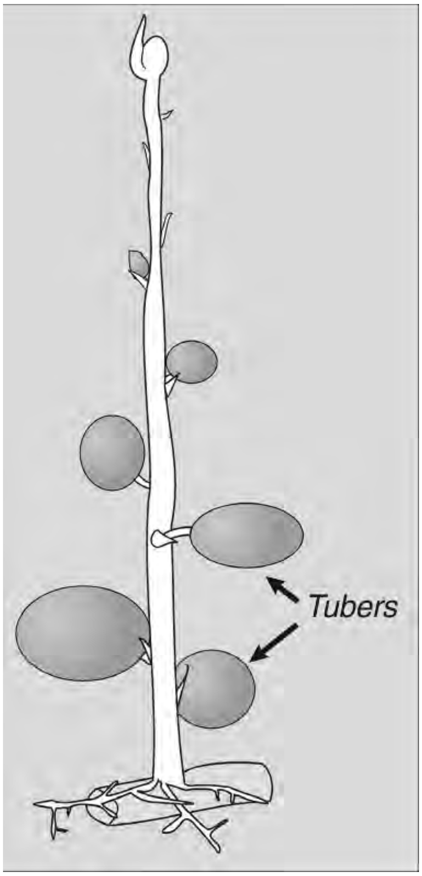

Fig. 2.6 Miniature tubers formed on an *in vitro* shoot of potato.

5. MICROGRAFTING

The transfer of small shoot apices onto rootstocks (termed micrografting), can be carried out *in vivo* or *in vitro*. Navarro (1988) lists four uses which have been found for the technique:

- Obtaining plants free from specific virus and virus-like diseases;
- A method for separating virus and virus-like organisms in mixed infections;
- For studying graft incompatibility between scions and rootstocks, and the histological and physiological aspects of grafting;

- A minimum risk method for importing plant material through quarantine.

Micrografting is thus indirectly useful in micropropagation: the necessary techniques are usually too time consuming and the proportion of successful takes is generally too low, for it to be of direct application.

The rootstocks used for micrografting are commonly newly-germinated seedlings, but it is also possible to use rooted cuttings or micropropagated shoots. Before micrografting can be carried out, it is necessary to prepare suitable rootstock material. When seedling rootstocks are used, and all stages of grafting are conducted *in vitro*, seeds are surface sterilised and germinated aseptically in vessels containing nutrient salts (e.g. those of MS medium). The seedlings may be supported on agar medium or on a porous substrate, such as sterile vermiculite, which allows the growth of a branched root system. Micrografting is then effected by cutting off the tops of the seedling rootstocks and placing small shoot apices onto the exposed surface. When grafts are successful, rootstock and scion grow together to produce a plant. It is usually necessary to examine freshly grafted seedlings on a regular basis and remove any adventitious shoot arising on or below the graft union.

Shoot tips to be grafted onto seedling rootstocks (i.e. the scions) are carefully excised from preferred plant material. In *Citrus* there has been most success when scions have been placed directly in one of two positions:

- into inverted T-shaped incisions immediately below the cut surface of a decapitated rootstock, or,
- onto the cambium layer or vascular ring of the cut surface (Fig 2.7).

Shoot or meristem tips intended for grafting can be taken from apical or axillary buds of actively growing shoots in the greenhouse or field, or may be removed from shoots growing *in vitro*. Once transferred, the survival of micrografted apices is partly dependent on their size. Very small apices must be used for virus elimination, making the technique difficult and unreliable. Tips 0.1-0.2 mm in length have been grafted for virus elimination from vines; with peach, slightly larger apices (0.5-1 mm) have been employed. The excision and transfer of very small shoot apices requires precise micro-manipulation under a binocular microscope. If large shoot apices are to be grafted, their bases are often cut into a wedge which is then inserted into a vertical cut on the rootstock (Fig 2.8).

Several techniques have been found to increase the proportion of successful graft unions:

- Tissue blackening, which commonly results in the death of very small scions, can be reduced by soaking explants in an anti-oxidant solution, and/or placing a drop of solution onto the severed rootstock immediately before inserting the scion. A solution of 2 g/l sodium diethyldithiocarbamate (DIECA) has been used for this purpose. Navarro (1988) advocates rapid manipulations to prevent phenolic oxidation and says that it is more effective than anti-oxidants.
- Apices to be grafted may be placed either directly onto a decapitated rootstock, or cultured for a short period before being transferred. There is often a better ‘take’ and more rapid growth if they are pre-cultured for a short while supported on paper above an MS mineral salt medium containing growth regulators. Jonard *et al.* (1983) found that adding a cytokinin to the medium (e.g. 0.1 mg/l zeatin if the apex is cultured for 48 h; 0.01 mg/l zeatin if the culture is continued for 48-240 h) was particularly effective in encouraging the rapid formation of leafy shoots once the graft has been made. An alternative is to place scion shoot tips into a growth regulator solution for a short period before grafting: a 5-10 minute immersion in either 10 mg/l 2, 4-D or 1 mg/l BA, doubled the number of successful micrografts of *Citrus* (Edriss and Burger, 1984). Starrantino and Caruso (1988) got a greater percentage of viable grafts when they dipped both shoot tips and the cut apex of young rootstocks in 0.5 mg/l BA for 20 min before the two were united. Yet another method is to place cytokinin (e.g. 2mg/l BA or, for peach, 10 mg/l zeatin) in a drop of water or agar gel between the scion and the rootstock.
- A greater proportion of graft unions may result from growing isolated meristem tips to a larger size before they are implanted. Isolated scion tips of peach have been cultured *in vitro* by the initial stages of meristem tip culture for a period of about two weeks until they have grown from 0.5-1 mm to ca. 10 mm.
- Desiccation is a major cause of the failure of graft unions. To prevent drying, Pliego-Alfaro and Murashige (1987) applied a layer of moist nutrient agar gel to connect the graft area with the medium. The gel had to be progressively removed from the top downwards during weeks 1-3 after grafting, or poor unions resulted.

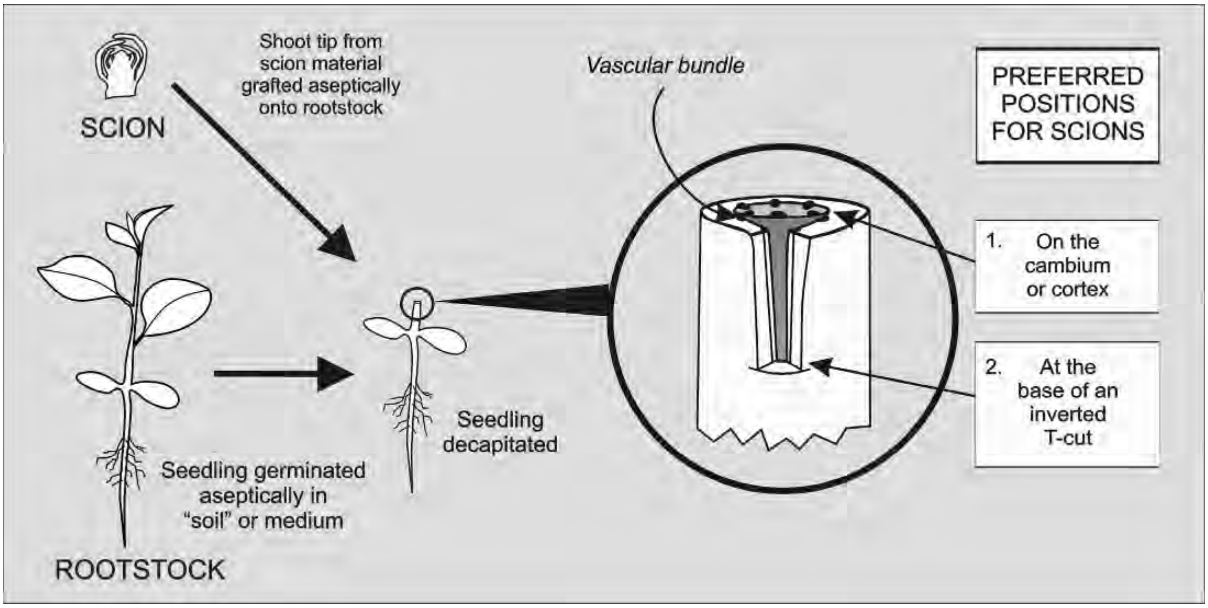

Fig. 2.7 Preferred positions for placing a shoot tip scion in the micrografting of *Citrus* [after Navarro *et al.,* 1975].

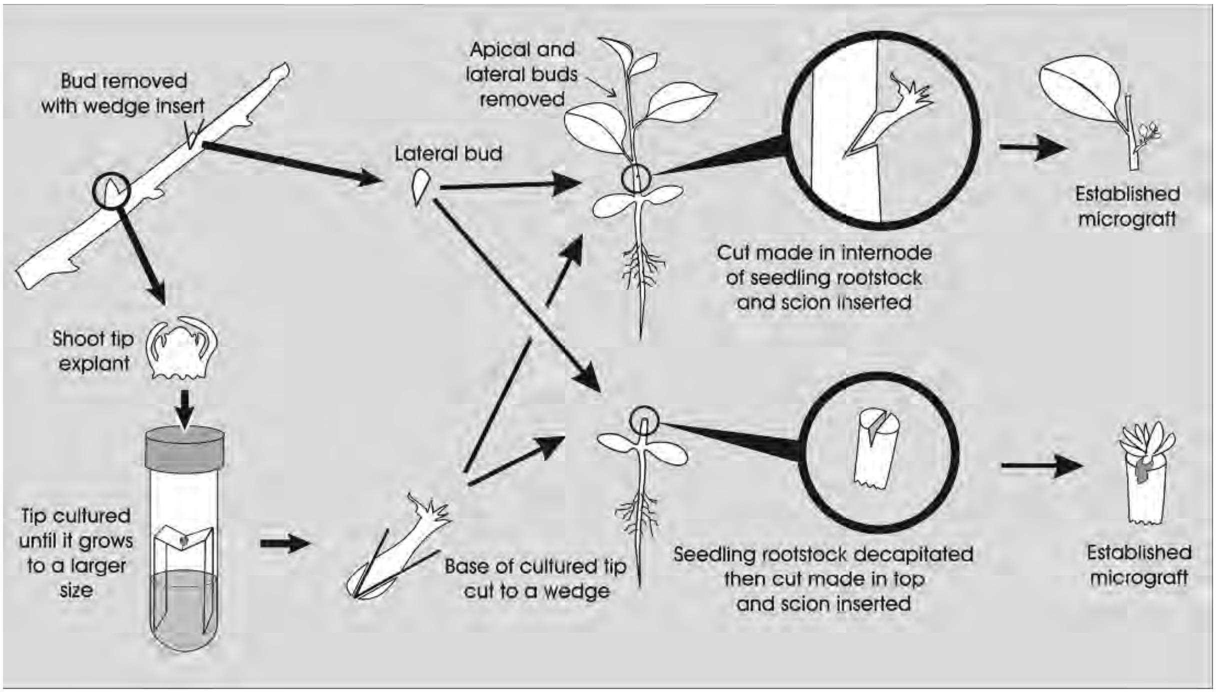

Fig. 2.8 Two alternative methods of micrografting with large scions [after Pliego-Alfaro and Murashige, 1987; Navarro, 1988].

When, as has been most common, micrografting has been carried out *in vitro*, considerable care needs to be taken over transferring grafted plants to the external environment. However, several authors have found that sterility is not necessary and that shoot apices can be united onto rootstocks grown *in vivo*. The proportion of completed grafts may be less than under aseptic conditions, but problems with eventual transfer are eliminated. A variety of scion material has been utilised for *in vivo* grafting, including

directly-excised 0.1-0.2 mm tips of *Citrus limon*, and meristem-cultured scion tips of peach. Once again, graft unions may be improved if cut surfaces are anointed with a DIECA (1g/l) plus cytokinin (10 g/l) solution (Jonard *et al.,* 1983). Plants are probably best kept in a growth room for a period after grafting, and desiccation of the graft union prevented by enclosing each plant in a plastic bag or by placing an elastic strip around the graft.

Grafting mature shoots of woody perennials onto juvenile rootstocks is known to induce juvenile characteristics in the resulting shoots, particularly if it is repeated successively (Chapter 11). Half of the plants resulting from micrografting adult lateral buds of avocado onto seedling rootstock were found to have some juvenile symptoms (Pliego-Alfaro and Murashige, 1987). But in *Citrus,* micrografting does not seem to induce juvenile characteristics, providing shoot tips are taken from an adult source: plants are thornless and come into flower rapidly (Navarro, 1988).

From having been developed as a method of producing virus-tested *Citrus*, micrografting is now widely used for the improvement of plants of this and related genera. It has also been practised on a wide range of other plants, primarily for virus-elimination (see reviews by Jonard *et al.,* 1983; Burger, 1985 and Navarro, 1988)

General reviews on micropropagation have been written by: Cassells (2005), Harney (1982), Holdgate (1977), Hussey (1978a, 1983), Jain *et al*. (2006), Lane (1982), Murashige (1974), and Rout and Jain (2004)

Books devoted completely or in great part to *in vitro* propagation are: Ahuja (1993), Conger (1981), Debergh and Zimmerman (1991), de Fossard (1981), Hall (1999), Hermann (1991-2006), Hvoslef-Eide and Preil (2005), Pierik (1997), Karata and Kozai (1992), Kyte (1983), Lumsden *et al*. (1994), Pennell (1987), Wetherell (1982), and Razdan (2003). A number of volumes of Acta Horticulturae deals specifically with micropropagation (Volumes 212, 225, 319, 530 and 616).

REFERENCES

ABO EL-NIL M.M. & HILDEBRANDT A.C. 1976 Cell wall regeneration and colony formation from isolated single geranium protoplasts in microculture. Can. J. Bot. 54, 1530-1534.

ABO EL-NIL M.M. & ZETTLER E.W. 1976 Callus initiation and organ differentiation from shoot tip cultures of *Colocasia esculenta* (Taro). Plant Sci. Lett. 6, 401-408.

ADAMS A.N. 1972 An improved medium for strawberry meristem culture. J. Hortic. Sci. 47, 263-264.

AHEE J., ARTHUIS P., CAS G., DUVAL Y., GUENIN G., HANOWER J., HANOWER P., LIEVOUX D., LIORET C., MALAURIE B., PANNETIER C., RAILLOT D., VARECHON C. & ZUCKERMAN L. 1981 La multiplication végétative *in vitro* du palmier huile par embryogenèse somatique. Oleagineux 36, 113-118.

AHUJA M.R. (ed.) 1993 *Micropropagation of Woody Plants*. Kluwer Academic Publishers. Dordrecht, Boston, London.

AMMIRATO P.V. & STEWARD F.C. 1971 Some effects of the environment on the development of embryos from cultured free cells. Bot. Gaz. 132, 149-158.

AMMIRATO P.V. 1973 Some effects of abscisic acid on the development of embryos from caraway cells in suspension culture. Am. J. Bot. 60, 4 (Suppl.) 22.

AMMIRATO P.V. 1974 The effects of abscisic acid on the development of somatic embryos from cells of caraway (*Carum carvi* L.). Bot. Gaz. 135, 328-337.

AMMIRATO P.V. 1976 Hormonal control of tuber formation in cultured axillary buds of *Dioscorea bulbifera* and *D. alata*. Plant Physiol 57, (Suppl.) 66.

AMMIRATO P.V. 1982 Growth and morphogenesis in cultures of the monocot. yam, *Dioscorea*. pp. 169-170 in Fujiwara A. (ed.) (q.v.).

ANDERSON W.C. & CARSTENS J.B. 1977 Tissue culture propagation of broccoli, *Brassica oleracea* (*Italica* group) for use in F1 hybrid seed production. J. Am. Soc. Hortic. Sci. 102, 69-73.

ARDITTI J., BALL E.A. & REISINGER D.M. 1977 Culture of flower-stalk buds: a method for vegetative propagation of *Phalaenopsis*. Am. Orchid Soc. Bull. 46, 236-240.

BALL E.A. 1946 Development in sterile culture of stem tips and subjacent regions of *Tropaeolum majus* L. and of *Lupinus albus* L. Am. J. Bot. 33, 301-318.

BALL E.A. & ARDITTI J. 1976 Node cultures as a means of clonal propagation for *Dendrobium*. Proc. 8th World Orchid Conf. 367-371.

BARGHCHI M. 1986b *In vitro* micropropagation of *Pistacia* rootstocks. Comb. Proc. Int. Plant Prop. Soc. 1985 35, 334-337.

BARLASS M. & SKENE K.G.M. 1978 *In vitro* propagation of grapevine (*Vitis vinifera* L.) from fragmented shoot apices. Vitis 17, 335-340.

BARLASS M. & SKENE K.G.M. 1980a Studies on the fragmented shoot apex of grapevine. I. The regenerative capacity of leaf primordial fragments *in vitro*. J. Exp. Bot. 31, 483-488.

BARLASS M. & SKENE K.G.M. 1980b Studies on the fragmented shoot apex of grapevine. II. Factors affecting growth and differentiation *in vitro*. J. Exp. Bot. 31, 489-495.

BARLASS M. & SKENE K.G.M. 1982a A scanning electron microscope study of adventitious bud formation in the grapevine. pp. 159-160 in Fujiwara A. (ed.) 1982 (q.v.).

BARLASS M. & SKENE K.G.M. 1982b *In vitro* plantlet formation from *Citrus* species and hybrids. Sci. Hortic. 17, 333-341.

BONGA J.M. 1976 Morphogenesis in cultures of Balsam fir. In Vitro 12, 333 (Abst. 158).

BOONNOUR K., WAINWRIGHT H. & HICKS R.G.T. 1988 The micropropagation of *Lonicera periclymenum* L. (Honeysuckle). Acta Hortic. 226, 183-189.

BRESSAN P.H., KIM Y-J., HYNDMAN S.E., HASEGAWA P.M. & BRESSAN R.A. 1982 Factors affecting *in vitro* propagation of rose. J. Am. Soc. Hortic. Sci. 107, 979-990.

BROWSE P.M. 1980 Propagation of plants from root cuttings. Plantsman 2, 54-62.

BURGER D.W. 1985 Micrografting: a tool for the plant propagator. Comb. Proc. Int. Plant Prop. Soc. 1984 34, 244-248.

BURGER D.W., LIU I. & WU I. 1985 Rapid micropropagation of *Paulownia tomentosa*. HortScience 20, 760-761.

BUTTON J. & KOCHBA J. 1977 Tissue culture in the citrus industry. pp. 70-92 in Reinert & Bajaj (eds.) 1977 (q.v.).

BUTTON J., KOCHBA J. & BORNMAN C.H. 1974 Fine structure of and embryoid development from embryogenic ovular callus of 'Shamouti' orange (*Citrus sinensis* Osb.). J. Exp. Bot. 25, 446-457.

CAPUANA M. & DEBERGH P. 1997 Improvement of the maturation and germination of horse chestnut somatic embryos. Plant Cell Tissue Organ Cult. 48, 23-29.

CASSELLS A. 2005 Principles of micropropagation. pp. 287-308 in Murch S.J. and Saxena P.K. (eds.) *Journey of a Single Cell to Plant*. Science Publishers, Enfield, Plymouth.

CHALUPA V. 1981 Clonal propagation of broad-leaved forest trees *in vitro*. Commun. Inst. Forest, Czechoslov. 12, 225-271.

CHALUPA V. 1983 *In vitro* propagation of willows (*Salix* spp.), European Mountain ash (*Sorbus aucuparia* L.) and Black Locust (*Robinia pseudoacacia* L.). Biol. Plant. 25, 305-307.

CHALUPA V. 1984a *In vitro* propagation of oak (*Quercus robur* L.) and Linden (*Tilia cordata* Mill.). Biol. Plant. 26, 374-376.

CHALUPA V. 1984b Vegetative propagation of English oak (*Quercus robur* L.), small-leaved Linden (*Tilia cordata* Mill.) and European mountain ash (*Sorbus aucuparia* L.) *in vitro*. Lesnictvi 30, 1019-1028.

CHALUPA V. 1992 Tissue culture propagation of black locust. pp.115-125 in Hanover J.W. (ed.) *Black Locust: Biology, Culture and Utilization*. Proc Int Conf., Publ. Dept. Forestry, Michigan State Univ.

CHAMPAGNAT M. & MOREL G. 1972 La culture *in vitro* des tissus de tubercules d'Ophrys. Compt. Rend. Acad. Sci. Paris 274D, 3379-3380.

CHATURVEDI H.C. & SHARMA A.K. 1977 Induced flowering in excised shoot apices of *Bougainvillea glabra* cv. 'Magnifica' grown *in vitro*. Ind. J. Exp. Biol. 15, 402-403.

CHATURVEDI H.C. & SHARMA A.K. 1985 Androgenesis in *Citrus aurantifolia* (Christm.) Swingle. Planta 165, 142-144.

CHATURVEDI H.C. & SINHA M. 1979 Mass propagation of *Dioscorea floribunda* by tissue culture. Econ. Bot. Inf. Service, Ext. Bull. National Bot. Res. Inst., Lucknow, 6.

CHEN Z. Chapter 19 – Rubber (*Hevea*). pp. 546-571 in Sharp W.R. *et al*. 1984 (eds.) *Handbook of Plant Cell Culture*. Vol. 2. *Crop Species*. Macmillan Publishing Co., New York, London.

CHENG T.-Y., SAKA H. & VOQUI-DINH T.H. 1980 Plant regeneration from soybean cotyledonary node segments in culture. Plant Sci. Lett. 19, 91-99.

CHU C.C., WANG Y.X. & SANG J.L. 1987 Long-term plant regeneration from callus cultures of haploid wheat. Plant Cell Tissue Organ Cult.11, 221-226.

CHURCHILL M.E., ARDITTI J. & BALL E.A. 1971 Clonal propagation of orchids from leaf tips. Am. Orchid Soc. Bull. 40, 109-113.

CHURCHILL M.E., BALL E.A. & ARDITTI J. 1973 Tissue culture of orchids. I. Methods for leaf tips. New Phytol. 72, 161-166.

CONGER B.V. (ed.) 1981 *Cloning Agricultural Plants via In Vitro Techniques*. CRC Press, Inc., Boca Raton, Florida.

COUMANS-GILLES M.F., KEVERS C., COUMANS M., CEULEMANS E. & GASPAR TH. 1981 Vegetative multiplication of sugarbeet through *in vitro* culture of inflorescence pieces. Plant Cell Tissue Organ Cult., 1, 93-101.

CRESSWELL R. & NITSCH C. 1975 Organ culture of *Eucalyptus grandis*. Planta 125, 87-90.

CRISP P. & WALKEY D.G.A. 1974 The use of aseptic meristem culture in cauliflower breeding. Euphytica 23, 305-313.

CURE W.W. & MOTT R.L. 1978 Anatomy of organogenesis in maize, wheat and oats. Physiol. Plant. 42, 91-96.

DALE P.J., THOMAS E., BRETTELL R.I.S. & WERNICKE W. 1981 Embryogenesis from cultured immature inflorescences and nodes of *Lolium multiflorum*. Plant Cell Tissue Organ Cult.1, 47-55.

DANDEKAR A.M., MARTIN L.A. & McGRANAHAN G. 1988 Genetic transformation and foreign gene expression in walnut tissue. J. Am. Soc. Hort. Sci. 113, 945-949.

DATTA K. & DATTA S.K. 1984 Rapid clonal multiplication of *Angelonia saricariaefolia* through tissue culture. Plant Cell Tissue Organ Cult. 3, 215-220.

DE FOSSARD R. A. 1976 *Tissue Culture for Plant Propagators.* Dept. Continuing Education, Univ. of New England, NSW 2351, Australia.

DE FOSSARD R.A., BARKER P.K. & BOURNE R.A. 1977 The organ culture of nodes of four species of *Eucalyptus*. Acta Hortic. 78, 157-165.

DE MAGGIO A.E. & WETMORE R.H. 1961 Growth of fern embryos in culture. Nature 191, 94-95.

DEBERGH P., ROGGEMANSD J., STANDAERT-DE METSENAERE R. 1990 Tissue culture in relation to ornamental plants. pp.161-189 in Bhojwani, S. S. (ed.) *Plant Tissue Culture: applications and limitations*. Elsevier, Amsterdam.

DEBERGH P.C. & MAENE L.J. 1981 A scheme for the commercial propagation of ornamental plants by tissue culture. Sci. Hortic. 14, 335-345.

DEBERGH P.C. & ZIMMERMAN R.H. 1991 *Micropropagation. Technology and Application.* Kluwer Academic Publishers. Dordrecht, Boston, London. ISBN 0-7923-0818-2

DIJAK M., SMITH K.L., WILSON T.J. & BROWN D.C.W. 1986 Stimulation of direct embryogenesis from mesophyll protoplasts of *Medicago sativa*. Plant Cell Rep. 5, 468-470.

DIXON R.A. & GONZALES R.A. 1995 *Plant Cell Culture: A Practical Approach*. Oxford University Press, Oxford. UK.

DODDS J.H. & ROBERTS L.W. 1995 *Experiments in Plant Tissue Culture.* Cambridge University Press, Cambridge, UK.

DONNELLY D. & VIDAVER W.E. 1988 *Glossary of plant tissue culture*. Belhaven Press, pp.141.

DOS SANTOS A.V.P., OUTKA D.E., COCKING E.C. & DAVEY M.R. 1980 Organogenesis and somatic embryogenesis in tissues derived from leaf protoplasts and leaf explants of *Medicago sativa*. Z. Pflanzenphysiol. 99, 261-270.

DRIRA N. & BENBADIS A. 1985 Multiplication végétative du palmier dattier (*Phoenix dactylifera* L.) par révision, en culture *in vitro*, d'ébauches florales de pieds femelles. J. Plant Physiol. 119, 227-235.

DUNSTAN D.I. & SHORT K.C. 1979a Shoot production from the flower head of *Allium cepa* L. Sci. Hortic. 10, 345-356.

DUNSTAN D.I. & SHORT K.C. 1977b *In vitro* studies on organogenesis and growth in *Allium cepa* tissue cultures. Acta Hortic. 78, 139-148.

EARLE E.D. & LANGHANS R.W. 1974c Propagation of *Chrysanthemum in vitro*. II. Production growth and flowering of plantlets from tissue cultures. J. Am. Soc. Hortic. Sci. 99, 352-358.

EDRISS M.H. & BURGER D.W. 1984 Micro-grafting shoot-tip culture of *Citrus* on three trifoliate rootstocks. Sci. Hortic. 23, 255-259.

EEUWENS C.J. & BLAKE J. 1977 Culture of coconut and date palm tissue with a view to vegetative propagation. Acta Hortic. 78, 277-281.

EICHHOLTZ D.A., ROBITAILLE H.A. & HASEGAWA P.M. 1979 Adventive embryony in apple. HortScience 14, 699-700.
EINSET J.W. & ALEXANDER J.H. (III) 1985 Multiplication of *Syringa* species and cultivars in tissue culture. Comb. Proc. Int. Plant Prop. Soc. 1984, 34, 628-636.
EL HASAN A.A. & DEBERGH P. 1987 *In vivo* tuberization of potato (*Solanum tuberosum* L.) plantlets freed *in vitro* from viruses. Meded. Fac. Landbouwwet. Rijksuniv. Gent 52, 1497-1500.
FIECHTER A. (ed.) 1980a *Advances in Biochemical Engineering. 16*. Springer Verlag, Berlin, Heidelberg, New York.
FIECHTER A. (ed.) 1980b *Advances in Biochemical Engineering. 18*. Springer Verlag, Berlin, Heidelberg, New York.
FLAMÉE M. & BOESMAN G. 1977 Clonal multiplication of *Phalaenopsis* hybrids by means of sections of the flower stalk. Meded. Fac. Landbouwwet. Rijksuniv. Gent 42, 1865-1868.
FREYSSINET M. & FREYSSINET G. 1988 Fertile plant regeneration from sunflower (*Helianthus annuus* L.) immature embryos. Plant Sci. 56, 177-181.
FUJIWARA A. (ed.) 1982 *Plant Tissue Culture* 1982. Proc. 5th. Int. Cong. Plant Tiss. Cell Cult., Japan. Assoc. Plant Tissue Culture, Japan.
GEIER T. & KOHLENBACH H.W. 1973 Entwicklung von Embryonen und embryogenem Kallus aus Pollenkornern von *Datura meteloides* und *Datura innoxia*. Protoplasma 78, 381-396.
GEIER T. 1986 Factors affecting plant regeneration from leaf segments of *Anthurium scherzerianum* Schott (Araceae) cultured *in vitro*. Plant Cell Tissue Organ Cult.6, 115-125.
GEIER T. 1987 Micropropagation of *Anthurium scherzerianum*: propagation schemes and plant conformity. Acta Hortic. 212, 439-443.
GEIER T. 1988 Ploidy variation in callus and regenerated plants of *Anthurium scherzerianum* Schott. Acta Hortic. 226, 293-298.
GENEVE R.L., KESTER S.T. & EL-SHALL S. 1990 *In vitro* shoot initiation in Kentucky coffee tree. HortScience 25, 578.
GHARYAL P.K. & MAHESHWARI S.C. 1981 *In vitro* differentiation of somatic embryoids in a leguminous tree - *Albizzia lebeck* L. Naturwiss. 68, 379-380.
GIELIS, J. & DEBERGH, P. 1998 *In vitro* flowering of bamboos. Flowering Newsl., November, 55-61
GIULIANO G., ROSELLINI D. & TERZI M. 1983 A new method for the purification of the different stages of carrot embryoids. Plant Cell Rep. 2, 216-218.
GOYAL Y., BINGHAM R.L. & FELKER P. 1985 Propagation of the tropical tree *Leucaena leucocephala* K67 by *in vitro* bud culture. Plant Cell Tissue Organ Cult. 4, 3-10.
GROUT B.W.W. & CRISP P. 1977 Practical aspects of the propa-gation of cauliflower by meristem culture. Acta Hortic. 78, 289-296.
GROUT B.W.W., WESTCOTT R.J. & HENSHAW G.G. 1977 Scanning electron microscope studies of multiple shoot tip production by meristem tip cultures of *Solanum curtilobum*. Ann. Bot. 41, 1113-1116.
HABERLACH G.T., COHEN B.A., REICHERT N.A., BAER M.A., TOWELL L.E. & HELGESON J.P. 1985 Isolation, culture and regeneration of protoplasts from potato and several related *Solanum* species. Plant Science 39, 67-74.
HACCIUS B. & HAUSNER G. 1976 A transplantable embryogenic callus from nucellar tissue of *Cynanchum vincetoxicum* and the significance of a globular proembryonal cell complex in the non-zygotic embryo development. Protoplasma 90, 265-282.
HACKETT W.P. & ANDERSON J.M. 1967 Aseptic multiplication and maintenance of differentiated carnation shoot tissue derived from shoot apices. Proc. Am. Soc. Hortic. Sci. 90, 365-369.
HALL R.D. (ed.) 1999 *Plant Cell Culture Protocols*. Humana Press, Ottowa.
HANDLEY L.W. & CHAMBLISS O.L. 1979 *In vitro* propagation of *Cucumis sativus* L. HortScience 14, 22-23.
HANDLEY L.W. & SINK K.C. 1985 Plant regeneration of *Solanum lycopersicoides* Dun. from stem explants, callus and suspension cultures. Plant Cell Tissue Organ Cult.5, 129-138.
HARAMAKI C. & MURASHIGE T. 1972 *In vitro* culture of *Gloxinia*. HortScience 7, 339.
HARAMAKI C. 1971 Tissue culture of *Gloxinia*. Comb. Proc. Int. Plant Prop. Soc. 21, 442-448.
HARNEY P.M. 1982 Tissue culture propagation of some herbaceous horticultural plants. pp. 187-208 in D.T, Ellis B.E., Harney P.M., Kasha K.J. & Peterson R.L. (eds.) *Application of Plant Cell and Tissue Culture to Agriculture and Industry.* Plant Cell Culture Centre, Univ. of Guelph, Ontario, Canada.
HAVEL L.F & NOVAK F.J. 1988 Regulation of somatic embryogenesis and organogenesis in *Allium carinatum* L. J. Plant Physiol. 132, 373-377.
HERMANN E.B. 1991-2006 Recent Advances in Plant Tissue Culture 1-10. see http://www.agritechpublications.com.
HILDEBRANDT V. & HARNEY P.M. 1983 *In vitro* propagation of *Syringa vulgaris* 'Vesper'. HortScience 18, 432-434.
HISAJIMA S. & CHURCH L. 1981 Multiple shoot formation from soybean embryo. Plant Physiol. 67, Suppl., 28.
HISAJIMA S. 1981 Multiple shoot formation from various kinds of plant seeds. Plant Physiol. 67, Suppl., 28.
HISAJIMA S. 1982a Multiple shoot formation from almond seeds and an excised single shoot. Agric. Biol. Chem. 46, 1091-1093.
HISAJIMA S. 1982b Multiple shoot formation from almond embryos. Biol. Plant. 24, 235-238.
HISAJIMA S. 1982c Microplant propagation through multiple shoot formation from seeds and embryos. pp. 141-142 in Fujiwara (ed.) 1982 (q.v.).
HODGE M.H. 1986 Propagation of herbaceous perennials by root cuttings. Comb. Proc. Int. Plant Prop. Soc. 1985 35, 548-554.
HOLDGATE D.P. 1977 Propagation of ornamentals by tissue culture. pp. 18-43 in Reinert and Bajaj (eds.) 1977 (q.v.).
HU C.-Y. & SUSSEX I.M. 1972 *In vitro* development of embryoids on cotyledons of *Ilex aquifolium*. Phytomorph. 21, 103-107.
HU C.-Y. 1977 Advances in *Ilex* embryo culture. Proc. 54th Meeting of the Holly Soc. of America 5-6 (HA 48 10780).
HU C.-Y., OCHS J.D. & MANCINI F.M. 1978 Further observations on *Ilex* embryoid production. Z. Pflanzenphysiol. 89, 41-49.
HUNAULT G. 1979 Recherches sur le comportement des fragments d'organes et des tissus de monocotyledones cultivés *in vitro*. V. Discussion Rev. Cytol. Biol. Vég. - Bot. 2, 259-287.
HUSSEY G. & GUNN H. 1983 Shoot regeneration *in vitro* in *Pisum*. Ann. Rep. John Innes Inst. 1981/1982, 127-128.
HUSSEY G. & GUNN H.V. 1984 Plant production in pea (*Pisum sativum* L. cvs. Puget and Upton) from long-term callus with superficial meristems. Plant Sci. Lett. 37, 143-148.
HUSSEY G. & HARGREAVES J. 1974 Multiplication of *in vitro* plantlets in bulbous species. Ann. Rep. John Innes Inst. 1974, 58-59.
HUSSEY G., HILTON J. & LUMSDEN P.1980 *In vitro* propagation of *Alstroemeria*. Ann. Rep. John Innes Inst. 1979, 56.
HUSSEY G. & STACEY N.J. 1981a *In vitro* propagation of potato (*Solanum tuberosum* L.) Ann. Bot. 48, 787-796.
HUSSEY G. & STACEY N.J. 1981b *In vitro* propagation of potato (*Solanum tuberosum* L.). Ann. Rep. John Innes Inst. 1980, 57-59.

HUSSEY G. 1978a *In vitro* propagation of the onion *Allium cepa* by axillary and adventitious shoot proliferation. Sci. Hortic. 9, 227-236.

HUSSEY G. 1978b The application of tissue culture to the vegetative propagation of plants. Science Prog. 65, 185-208.

HUSSEY G. 1983 *In vitro* propagation of horticultural and agricultural crops. pp. 111-138 in Mantell & Smith (eds.) *Plant Biotechnology*. Soc. Exp. Biol. seminar series 18 1983. C.U.P.

HUTCHINSON J.F. 1985 Effect of explant type on shoot proliferation and physical support on root initiation for a range of horticultural species. pp. 327-328 in Henke R.R., Hughes, K.W., Constantin M.J., Hollaender A.A. and Wilson C.M. (eds.) *Tissue Culture in Forestry and Agriculture*. Plenum Press, New York, London.

HVOSLEF-EIDE A.K. & PREIL W. (eds.) 2005 *Liquid Systems for In Vitro Plant Propagation*. Springer-Verlag, New York.

INOUE M. & MAEDA E. 1982 Control of organ formation in rice callus using two-step culture method. pp. 183-184 in Fujiwara A. (ed.) 1982 (q.v.).

JACKSON J.A. & DALE P.J. 1988 Callus induction, plant regeneration and an assessment of cytological variation in regenerated plants of *Lolium multiflorum* L. J. Plant Physiol. 132, 351-355.

JAGADISH CHANDRA K.S. & SREENATH H.L. 1982 *In vitro* culture and morphogenetic studies in some species of *Cymbopogon* Spreng. (aromatic grasses). pp. 703-705 in Fujiwara A. (ed.). (q,v.).

JAIN S.M., JENKS M.A., ROUT G.R. & RADEJOVIĆ L. 2006 Micropropagation of ornamental potted plants. Prop. Orn. Plants 6, 67-82.

JAIN S.M., SOPORY S.K. & VEILLEUX R.E. (eds) 1996 & 1997 *In Vitro Haploid Production in Higher Plants*, 5 volumes. Kluwer Academic Publishers, Dordrecht, The Netherlands

JOHN, A. & MURRAY, B.W. 1981 Micropropagation of Sitka spruce (*Pice sitchensis*). pp. 65-70 in AFOCEL *Colloque International sur la Culture in vitro des Essences Forestières*. AFOCEL Fontainebleau Publ.

JONARD R., HUGARD J., MACHEIX J.-J., MARTINEZ J., MOSELLA-CHANCEL L., POESSEL J.L. & VILLEMUR P. 1983 *In vitro* micrografting and its application to fruit science. Sci. Hortic. 20, 147-159.

KING P.J., POTRYKUS I. & THOMAS E. 1978 *In vitro* genetics of cereals. Problems and perspectives. Phyiol. Veg. 16, 381-399.

KING P. & SHIMAMOTO K. 1984 Chapter 3 - Maize. pp. 69-91 in Sharp W.R. *et al*. 1984 (eds.) *Handbook of Plant Cell Culture*. Vol. 2. *Crop Species*. Macmillan Publishing Co., New York, London.

KONONOWICZ H. & JANICK L. 1984 *In vitro* propagation of *Vanilla planifolia*. HortScience 19, 58-59.

KNAUSS J.F. 1976 A partial tissue culture method of pathogen-free propagation of selected ferns from spores. Proc. Fla. State Hort. Soc. 89, 363-365.

KOCHBA J. & BUTTON J. 1974 The stimulation of embryo-genesis and embryoid development in habituated ovular callus from the 'Shamouti' orange (*Citrus sinensis*) as affected by tissue age and sucrose concentration. Z. Pflanzenphysiol. 73, 415-421.

KONAR R.N. & NATARAJA K. 1965a Experimental studies in *Ranunculus sceleratus* L. Development of embryos from the stem epidermis. Phytomorph. 15, 132-137.

KONAR R.N. & NATARAJA K. 1965b Experimental studies in *Ranunculus sceleratus* L. plantlets from truly suspended cells and cell groups. Phytomorph. 15, 206-211.

KONAR R.N. & OBEROI Y.P. 1965 *In vitro* development of embryoids on the cotyledons of *Biota orientalis*. Phytomorph. 15, 137-140.

KONAR R.N., THOMAS E. & STREET H.E. 1972a Origin and structure of embryoids arising from epidermal cells of the stem of *Ranunculus sceleratus* L. J. Cell Sci. 11, 77-93.

KOZAI, T. & SMITH, M. A. L. 1995 Environmental control in plant tissue culture. General introduction and overview. pp. 301-318 in Aitken-Christie, J., Kozai, T. & Smith, M. A. L (eds.) *Automation and Environmental Control in Plant Tissue Culture.* Kluwer Academic Publishers, The Netherlands.

KRIKORIAN A.D. & KANN R.P. 1979 Clonal micropropagation of daylilies. pp. 835-836 in Sharp *et al.*(eds.) 1979 (q.v.).

KRISTENSEN H.R. 1984 Potato tissue culture. pp. 25-49 in *Micropropagation of selected root crops, palms, citrus and ornamental species*. FAO Plant Production and Protection Paper 59, FAO, Rome. ISBN 92-5-102157-0.

KURATA K. & KOZAI T. 1992 *Transplant Production Systems*. Kluwer Academic Publishers. Dordrecht, Boston, London.

KURATA, K. 1995 Image analysis for embryogenesis. pp.165-186 in Aitken-Christie J, Kozai T. and Smith M.A.L. (eds.) *Automation and Environmental Control in Plant Tissue Culture.* Kluwer Academic Publ., The Netherlands

KYTE L. & BRIGGS B. 1979 A simplified entry into tissue culture production of rhododendrons. Comb. Proc. Int. Plant Proc. Soc. 29, 90-95.

KYTE L. 1983 *Plants from Test Tubes. An Introduction to Micropropagation*. The Timber Press, Beaverton, Oregon. ISBN 0-917304-50-0.

LA RUE C.D. 1936 The growth of plant embryos in culture. B. Torrey Bot. Club 63, 365-382.

LANE W.D. 1979 Regeneration of pear plants from shoot meristem tips. Plant Sci. Lett. 16, 337-342.

LANE W.D. 1982 Plant manipulation *in vitro* with hormones. Comb. Proc. Int. Plant Prop. Soc. 1981 31, 101-108.

LEVIN, R., ALPER. Y., STAV, R., & WATAD A. A. 1997 Methods and apparatus for liquid media and semi-automated micropropagation. Acta Hortic. 447, 659-663

LEVY D. 1988 Propagation of potato by the transfer of transplants of *in vitro* proliferated shoot cuttings into the field. Scientia Hort. 36, 165-171.

LI, Z., TRAORE, A., MAXIMOVA, S. & GUILTINAN, M. J. 1998 Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. In Vitro Cell. Dev. - Pl. 34, 293-299.

LIN K.C., CHEN F.C. & WANG P.J. 1978 J. Chinese Soc. Hort. Sci. 24, p. 260.

LINDEMANN E. 1984 System and apparatus for plant tissue culture propagation. US Gov. patent no. 4463522.

LIPPMANN B. & LIPPMANN G. 1984 Induction of somatic embryos in cotyledonary tissue of soybean, *Glycine max* L. Merr. Plant Cell Rep. 3, 215-218.

LITZ R.E. & CONOVER R.A. 1981a *In vitro* polyembryony in *Carica papaya* ovules. HortScience 16, 459.

LITZ R.E. & CONOVER R.A. 1981b *In vitro* polyembryony in *Carica papaya* L. ovules. Z. Pflanzenphysiol. 104, 285-288.

LITZ R.E. 1987 Application of tissue culture to tropical fruits. pp. 407-418 in Green C.E, Sommers D.A., Hackett W.P. & Biesboer D.D. (eds.) *Plant Tissue and Cell Culture*. Alan R. Liss, Inc., New York.

LITZ R. & JAISWAL V.S. 1991 Micropropagation of tropical and subtropical fruits. pp. 247-263 in Debergh, P. and Zimmerman, R. (eds.) *Micropropagation: Technology and Application*. Kluwer Academic Publishers, The Netherlands.

LOO S.-W. 1945 Cultivation of excised stem tips of *Asparagus in vitro*. Am. J. Bot. 32, 13-17.

LOO S.-W. 1946a Further experiments on the culture of excised asparagus tips *in vitro*. Am. J. Bot. 33, 156-159.

LOO S.-W. 1946b Cultivation of excised stem tips of dodder *in vitro*. Am. J. Bot. 33, 295-300.

LOYOLA-VARGAS V.M. & VAZQUEZ-FLOTA F. 2005 *Plant Cell Culture Protocols*. Humana Press, New York.

LU C.-Y. & VASIL I.K. 1981a Somatic embryogenesis and plant regeneration from leaf tissues of *Panicum maximum* Jacq. Theor. Appl. Genet. 59, 275-280.

LU C.-Y. & VASIL I.K. 1981b Somatic embryogenesis and plant regeneration from freely-suspended cells and cell groups of *Panicum maximum*. Ann. Bot. 48, 543-548.

LUMSDEN P.J., NICHOLAS J.R. & DAVIES W.J. (eds.) 1994 *Physiology, Growth and Development of Plants in Culture*. Kluwer Academic Publishers. Dordrecht, Boston, London.

LUPOTTO E. 1986 The use of single somatic embryo culture in propagating and regenerating lucerne (*Medicago sativa* L.). Ann. Bot. 57, 19-24.

MACKAY W.A. & KITTO S.L. 1988 Factors affecting *in vitro* shoot proliferation of French tarragon. J. Am. Soc. Hortic. Sci. 113, 282-287.

MAHESHWARI P. & BALDEV B. 1961 Artificial production of buds from the embryos of *Cuscuta reflexa*. Nature 191, 197-198.

MAHESWARAN G. & WILLIAMS E.G. 1985 Origin and development of somatic embryoids formed directly on immature embryos of *Trifolium repens in vitro*. Ann. Bot. 56, 619-630.

MAPES M.O. 1973 Tissue culture of bromeliads. Comb. Proc. Int. Plant Prop. Soc. 23, 47-55.

MARGARA J. 1969a Étude des facteurs de la néoformation de bourgeons en culture *in vitro* chez le chou-fleur (*Brassica oleracea* L. var. *botrytis*). Ann. Physiol. Veg. 11, 95-112.

MARGARA J. 1969b Étude préliminaire des facteurs de la néoformations de bourgeons chez le chou-fleur (*Brassica oleracea* L. var. *botrytis*). Compt. Rend. Acad. Sci. Paris 268D, 686-688.

MARGARA J. 1969c Sur l'aptitude de tissus au bourgeonnement et à l'initiation florale *in vitro*. Compt. Rend. Acad. Sci. Paris 268D, 803-805.

MARGARA J. 1977a Effets d'auxines et d'antiauxines sur la néoformation de bourgeons *in vitro* chez le Chou-fleur (*Brassica oleracea* L. var. *botrytis*). Compt. Rend. Acad. Sci. Paris 284D, 1883-1885.

MARIN J.A. & GELLA R. 1987 Aclimatization of the micro-propagated cherry rootstock 'Masto de Montanana' (*Prunus cerasus* L.). Acta Hortic. 212, 603-606.

MARKS T.R. & MEYERS P. E. 1994 Physiological variability arising from *in vitro* is induced by shoot selection and manipulation strategies. J. Hortic. Sci. 69, 1-9

McCELLAND M.T. & SMITH M.A.L. 1990 Vessel type, closure, and explant orientation influence *in vitro* performance of five woody species. HortScience 25, 797-800.

McCOWN B.H. & AMOS R. 1982 A survey of the response of gymnosperms to microculture. HortScience 17, 489.

MCCOWN B.H., ZELDIN E. L., PINKALLA H. A. & DEDOLPH R.R. 1988 Nodule culture: a developmental pathway with high potential for regeneration, automated micropropagation and plant metabolite production from woody plants. pp. 49-66 in Hanover J.W. and Keathley D.E. (eds.) *Genetic Manipulation of Woody Plants*. Plenum Press, New York & London.

McGRANAHAN G.H., LESLIE C.A., URATSU S.L., MARTIN L.A. & DANDEKAR A.M. 1988 *Agrobacterium*-mediated transformation of walnut somatic embryos and regeneration of transgenic plants. Nature Biotechnol. 6, 800-804.

McWILLIAM A.A., SMITH S.M. & STREET H.E. 1974 The origin and development of embryoids in suspension cultures of carrot (*Daucus carota*). Ann. Bot. 38, 243-250.

MISRA P. & CHATURVEDI H.C. 1984 Micropropagation of *Rosmarinus officinalis* L. Plant Cell Tissue Organ Cult. 3, 163-168.

MILLER C.O. 1961b Kinetin related compounds in plant growth. Ann. Rev. Plant Physiol. 12, 395-408.

MILLER J.H. 1968 Fern gametophytes as experimental material. Bot. Rev. 34, 361-426.

MIURA Y. & TABATA M. 1986 Direct somatic embryogenesis from protoplasts of *Foeniculum vulgare*. Plant Cell Rep. 5, 310-313.

MIYAGAWA H., FUJIOKA N., KOHDA H., YAMASAKI K., TANIGUCHI K. & TANAKA R. 1986 Studies on the tissue culture of *Stevia rebaudiana* and its components; II. Induction of shoot primordia. Planta Med. 52, 321-323.

MOREL G. 1960 Producing virus-free *Cymbidium*. Am. Orchid Soc. Bull. 29, 495-497.

MOREL G. 1964 Tissue culture - a new means of clonal propagation in orchids. Am. Orchid Soc. Bull. 33, 473-478.

MOTT R.L. 1981 Trees. pp. 217-254 in Conger B.V. (ed.) *Cloning Agricultural Plants via In Vitro Techniques*. CRC Press Inc., Boca Raton, Florida.

MURASHIGE T. & SKOOG F. 1962 A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15, 473-497.

MURASHIGE T. 1974 Plant propagation through tissue cultures. Ann. Rev. Plant Physiol. 25, 135-166.

NASIR F.R. & MILES N.W. 1981 Histological origin of EMLA 26 apple shoots generated during micropropagation. HortScience 16, 417.

NAVARRO L. 1988 Application of shoot-tip grafting *in vitro* to woody species. Acta Hortic. 227, 43-55.

NAVARRO L., ROISTACHER C.N. & MURASHIGE T. 1975 Improvement of shoot-tip grafting *in vitro* for virus-free citrus. J. Am. Soc. Hortic. Sci. 100, 471-479.

NG S.Y.C. 1988 *In vitro* tuberisation in white yam (*Dioscorea rotundata* Poir.) Plant Cell Tissue Organ Cult. 14, 121-128.

NORSTOG K. & RHAMSTINE E. 1967 Isolation and culture of haploid and diploid *Cycad* tissues. Phytomorph. 17, 374-381.

NORSTOG K. 1961 The growth and differentiation of cultured barley embryos. Am. J. Bot. 48, 876-884.

NORSTOG K. 1965a Development of cultured barley embryos. I. Growth of 0.1-0.4 mm embryos. Am. J. Bot. 52, 538-546.

NORSTOG K. 1965b Induction of apogamy in megagametophytes of *Zamia integrifolia*. Am. J. Bot. 52, 993-999.

NORSTOG K. 1970 Induction of embryo-like structures in cultured barley embryos. Dev. Biol. 23, 665-670.

NORSTOG K. 1979 Embryo culture as a tool in the study of comparative and developmental morphology. pp. 179-202 in Sharp *et al.* (eds.) 1979 (q.v.).

ORTON T.J. 1985 Genetic instability during embryogenic cloning of celery. Plant Cell Tissue Organ Cult. 4, 159-169.

PAPACHATZI M., HAMMER P.A. & HASEGAWA P.M. 1981 *In vitro* propagation of *Hosta decorata* 'Thomas Hogg' using cultured shoot tips. J. Am. Soc. Hortic. Sci. 106, 232-236.

PARANJOTHY K. & ROHANI O. 1982 *In vitro* propagation of oil palm. pp. 747-749 in Fujiwara A. (ed.) 1982 (q.v.).

PENCE V.C., HASEGAWA P.M. & JANICK J. 1980a Initiation and development of asexual embryos of *Theobroma cacao in vitro*. Z. Pflanzenphysiol. 98, 1-14.

PENCE V.C., PETRETIC N.L., HASEGAWA P.M. & JANICK J. 1980b Regulation of fatty acid synthesis in asexual embryos of cacao. Plant Physiol. 65, Suppl., 38.

PENNELL D. 1987 *Micropropagation in Horticulture*. Grower Books, London. ISBN 0 901361 83 6

PETRÙ E. 1970 Development of embryoids in carrot root callus culture (*Daucus carota* L.). Biol. Plant. 12, 1-5.

PIEPER M.A. & SMITH M.A.I. 1988 A whole plant microculture selection system for Kentucky bluegrass. Crop Sci. 28, 611-614.
PIERIK R.L.M. 1997 *In Vitro Culture of Higher Plants.* Kluwer Academic Publishers, Dordrecht, The Netherlands.
PIERON S., BELAIZI M. & BOXUS P. 1993 Nodule culture, a possible morphogenetic pathway in *Cichorium intybus* L. propagation. Sci. Hortic. 53, 1-11
PLIEGO-ALFARO F. & MURASHIGE T. 1987 Possible rejuvenation of adult *Avocado* by graftage onto juvenile rootstocks *in vitro*. HortScience 22, 1321-1324.
PLISSIER B., BOUCHEFRA O., PEPIN R. & FREYSSINET G. 1990 Production of isolated somatic embryos from sunflower thin cell layers. Plant Cell Rep. 9, 47-50.
POW J.J. 1969 Clonal propagation *in vitro* from cauliflower curd. Hort. Res. 9, 151-152.
POWLING A. & HUSSEY G. 1981 Establishment of aseptic cultures of sugar beet. Ann. Rep. John Innes Inst. 1980, p. 60.
PRETOVA A. & WILLIAMS E.G. 1986 Direct somatic embryogenesis from immature zygotic embryos of flax (*Linum usitatissimum* L.). J. Plant Physiol. 126, 155-161.
RADOJEVIC L. 1988 Plant regeneration of *Aesculus hippocastanum* L. (Horse Chestnut) through somatic embryogenesis. J. Plant Physiol. 132, 322-326.
RADOJEVIC L. 1995 Somatic embryogenesis in horse chestnut (*Aesculus hippocastanum* L.). pp. 409-444 in Jain S.M., Gupta P. & Newton R. (eds.) *Somatic Embryogenesis in Woody Plants, Vol 2* Kluwer Academic Publ., Dordrecht, The Netherlands.
RAJASEKARAN K. & MULLINS M.G. 1979 Embryos and plantlets from cultured anthers of hybrid grapevines. J. Exp. Bot. 30, 399-407.
RANGASWAMY N.S. 1982 Nucellus as an experimental system in basic and applied tissue culture research. pp. 269-286 in Rao A.N. (ed.) 1982 *Tissue Culture of Economically Important Plants.* Proc. Int. Symp., Singapore, 1981. COSTED and ANBS Publications.
RASHID A. & STREET H.E. 1973 The development of haploid embryoids from anther cultures of *Atropa belladonna* L. Planta 113, 263-270.
RAZDAN M.K. 2003 *Introduction to Plant Tissue Culture.* Science Publishers, Inc., Enfield, Plymouth.
REINERT J., BACKS-HSEMANN D. & ZERMAN H. 1971 Determination of embryo and root formation in tissue cultures from *Daucus carota*. pp. 261-268 in *Les Cultures de Tissus de Plantes*. Coll. Int. CNRS 193 Paris.
REINERT J. & BAJAJ Y. P. S. (eds.) 1977 *Plant Cell, Tissue and Organ Culture*. Springer-Verlag, Berlin.
REUTHER G. 1990. Histological and cytological selection of somaclonal and induced variants of asparagus derived from callus cultures. Acta Hortic. 280, 385-393.
ROBBINS W.J. 1922 Cultivation of excised root tips and stem tips under sterile conditions. Bot. Gaz. 73, 376-390.
ROCA W.M., ESPINOZA N.O., ROCA M.R. & BRYAN J.E. 1978 A tissue culture method for the rapid propagation of potatoes. Am. Pot. J. 55, 691-701.
RODRIGUEZ R. 1982 Stimulation of multiple shoot-bud formation in walnut seeds. HortScience 17, 592.
ROUT G.R. & JAIN S.M. 2004 Micropropagation of ornamental plants – cut flowers. Prop. Orn. Plants 4, 3-28.
RUGINI E. & FONTANAZZA G. 1981 *In vitro* micropropagation of ‘Dulce Agogia’ olive. HortScience 16, 492-493.
SABHARWAL P.S. 1963 *In vitro* culture of ovules, nucelli and embryos of *Citrus reticulatq* Blanco var. Nagpuri. pp.265-273 in: Maheshawari P. and Rangaswamy N.S. (eds.). *Plant Tissue and Organ Culture.* Int. Soc. Plant Morphologists, Univ. Delhi, India.
SACHS T. & THIMANN K.V. 1964 Release of lateral buds from apical dominance. Nature 201, 939-940.
SAUNDERS J.W. & BINGHAM E.T. 1972 Production of alfalfa plants from callus tissue. Crop Sci. 12, 804-808.
SAUTON A., MOURAS A. & LUTZ A. 1982 Plant regeneration from citrus root meristems. J. Hortic. Sci. 57, 227-231.
SCHWAIGER G. & HORN W. 1988 Somaclonal variations in micropropagated *Kalanchoe* hybrids. Acta Hortic. 226, 695-698.
SIHACHAKR D., HAICOUR R., SERRAF I., BARRIENTOS F., HERBRETEAU C., DUCREUX G., ROSSIGNOL L. & SOUVANNAVONG V. 1988 Electrofusion for the production of somatic hybrid plants of *Solanun melongena* L. and *Solanum khasianum* C.B. Clark. Plant Science 57, 215-223.
SHAH R.R. & DALAL K.C. 1980 *In vitro* multiplication of *Glycyrrhiza*. Current Sci. 49, 69-71.
SHAH R.R. & DALAL K.C. 1982 *Glycyrrhiza glabra* (liquorice) from test tube to field. pp. 685-686 in Fujiwara A. (ed.) (q.v).
SHARP W.R., LARSEN P.O., PADDOCK E.F. & RAGHAVAN V. (eds.) 1979 *Plant Cell and Tissue Culture*. Ohio State Univ. Press, Columbus.
SHARP W.R., SONDAHL M.R., CALDAS L.S. & MARAFFA S.B. 1980 The physiology of *in vitro* asexual embryogenesis. Hort. Rev. 2, 268-310.
SHEN X.S. & MULLINS M.G. 1984 Propagation *in vitro* of pear, *Pyrus communis* L, cultivars William's Bon Chretien, Packham's Triumph and Beurre Bosc. Sci Hortic 23, 51-57.
SHU O. WANG & SU SHIEN MA 1978 Clonal multiplication of *Chrysanthemum in vitro*. J. Agric. Assoc. China 101, 64-73.
SIM G.-E., LOH C.-H. & GOH C.-J. 1988 Direct somatic embryogenesis from protoplasts of *Citrus mitis* Blanco. Plant Cell Rep. 7, 418-420.
SINGH F. & PRAKASH D. 1984 *In vitro* propagation of *Thunia alba* (Wall.) Reichb. F. through flower stalk cuttings. Scientia Hort 24. 385-390.
SKOOG F., STRONG F.M. & MILLER C.O. 1965 Cytokinins. Science 148, 532-533.
SMITH D.L. & KRIKORIAN A.D. 1988 Production of somatic embryos from carrot tissues in hormone-free medium. Plant Sci. 58, 103-110.
SMITH D.L. & KRIKORIAN A.D. 1990 pH control of carrot somatic embryogenesis. pp. 449-453 in Nijkamp H.J.J., Van Der Plas I.H.W. and Van Aartrijk (eds.) *Progress in Plant Cellular and Molecular Biology.* Proc. VIIth Int. Cong. on Plant Tissue and Cell Culture. Amsterdam, The Netherlands. 24-29 June 1990. Kluwer Academic Publishers, Dortrecht, Netherlands.
SNIR I. 1984 *In vitro* propagation of ‘Canino’ apricot. HortScience 19, 229-230.
SPIEGEL-ROY P. & KOCHBA J. 1980 Role of polyembryony and apomixis in *Citrus* propagation and breeding. pp. 28-48 in Fiechter A. (ed.) 1980a (q.v.).
STAMP J.A. & HENSHAW G.G. 1982 Somatic embryogenesis in cassava. Z. Pflanzenphysiol. 105, 183-187.
STAMP J.A. & MEREDITH C.P. 1988 Proliferative somatic embryogenesis from zygotic embryos of grapevine. J. Am. Soc. Hortic. Sci. 113, 941-945.
STANDARDI A. & CATALANO F. 1985 Tissue culture propagation of kiwi fruit. Comb. Proc. Int. Plant Prop. Soc. 1984 34, 236-243.
STANDARDI A. 1982 Effetti di sobcolture ripetute in germogli di *Actinidia sinensis* (Pl.) coltivati *in vitro*. Riv. Ortoflorofrutti. Ital. 66, 419-429.
STARRANTINO A. & CARUSO A. 1988 The shoot-tip grafting technique applied to citriculture. Acta Hortic. 227, 101-103.
STREET H.E. 1979 Embryogenesis and chemically induced organogenesis. pp. 123-153 in Sharp *et al.* (eds.) 1979 (q.v.).

SUNDERLAND N. & DUNWELL J.M. 1977 Anther and pollen culture. pp. 223-265 in Street H.E. (ed.) *Plant Tissue and Cell Culture*. Bot. Monographs Vol.11, Blackwell Scientific Publications. Oxford, London.

SUTTER E. & LANGHANS R.W. 1979 Epicuticular wax formation on carnation plantlets regenerated from shoot tip culture. J. Am. Soc. Hortic. Sci. 104, 493-496.

SUTTER E. & LANGHANS R.W. 1980 Formation of epicuticular wax and its effect on water loss in cabbage plants regenerated from shoot tip culture. HortScience 15, 429 (Abst. 428).

TANAKA M., HASEGAWA A. & GOI M. 1975 Studies on the clonal propagation of monopodial orchids by tissue culture: 1. Formation of protocorm-like bodies (PLB) from leaf tissue in above sp. J. Jap. Soc. Hort. Sci. 44, 47-58.

TANAKA M. & SAKANISHI Y. 1978 Factors affecting the growth of *in vitro* cultured lateral buds from *Phalaenopsis* flower stalks. Scientia Hort. 8, 169-178.

THOMAS E., HOFFMANN F., POTRYKUS I. & WENZEL G. 1976 Protoplast regeneration and stem embryogenesis of haploid androgenetic rape. Mol. Gen. Genet. 145, 245-247.

TIDEMAN J. & HAWKER J.S. 1982 *In vitro* propagation of latex-producing plants. Ann. Bot. 49, 273-279.

TOPOONYANONT N. & DILLEN W. 1988 Capitulum explants as a start for micropropagation of *Gerbera*. − Culture technique and applicability. Meded. Fac. Landbouww. Univ. Gent 53, 169-173.

TOPOONYANONT N., AMPAWAN R. & DEBERGH P. C. 1999 Reversion of *Limonium* hybrid 'Misty Blue' inflorescence development and its applicability in micropropagation. Sci. Hort. 83, 283-299.

TOSHINORI A. & FUTSUHARA Y. 1985 Efficient plant regeneration by somatic embryogenesis from root callus tissues of rice (*Oryza sativa* L.). J. Plant Physiol. 121, 111-118.

TRIGIANO R.N. & GRAY D.J. 2004 *Plant Development and Biotechnology*. CRC Press, Roca Baton.

TRIMBOLI D.S., PRAKASH N. & DE FOSSARD R.A. 1977 The initiation, rooting and establishment of cortical buds in cauliflower. Acta Hortic. 78, 243-248.

TSAY H.S. & SU C.Y. 1985 Anther culture of papaya (*Carica papaya* L.). Plant Cell Rep. 4, 28-30.

TULECKE W. & McGRANAHAN G. 1985 Somatic embryogenesis and plant regeneration from cotyledons of walnut, *Juglans regia* L. Plant Sci. 40, 57-63.

VAN DIJK, P. & VAN DAMME, J. 2000 Apomixis technology and the paradox of sex. Trends Plant Sci. 5, 81-84.

VASIL I.K. & HILDEBRANDT A.C. 1966 Variations of morphogenetic behaviour in plant tissue cultures. II. *Petroselinum hortense*. Am. J. Bot. 53, 869-874.

VASIL V. & VASIL I.K. 1981a Somatic embryogenesis and plant regeneration from tissue cultures of *Pennisetum americanum* and *P. americanum* x *P. purpureum* hybrid. Am. J. Bot. 68, 864-872.

VASIL V. & VASIL I.K. 1981b Somatic embryogenesis and plant regeneration from suspension cultures of pearl millet (*Pennisetum americanum*). Ann. Bot. 47, 669-678.

VIEITEZ A.M. & VIEITEZ M.L. 1980 Culture of chestnut shoots from buds *in vitro*. Plant Physiol 55, 83-84.

VINE S.J. & JONES O.P. 1969 The culture of shoot tips of hop (*Humulus lupulus*) to eliminate viruses. J. Hortic. Sci. 44, 281-284.

WAINWRIGHT H. & ENGLAND N. 1987 The micropropagation of *Prosopis juliflora* (Swartz.): Establishment *in vitro*. Acta Hortic. 212, 48-53.

WAINWRIGHT H. & MARSH J. 1986 The micropropagation of watercress (*Rorippa nasturtium-aquaticum* L.). J. Hort. Sci. 61, 251-256.

WALKEY D.G.A. & WOOLFITT J.M.G. 1968 Clonal multiplication of *Nicotiana rustica* from shoot meristems in culture. Nature 220, 1346-1347.

WANG D.-Y. & VASIL I.K. 1982 Somatic embryogenesis and plant regeneration from inflorescence segments of *Pennisetum purpureum* Schum. (Napier or Elephant grass). Plant Sci. Lett. 25, 147-154.

WANG P. J. & HU C. Y. 1980 Regeneration of virus-free plants through *in vitro* culture. pp. 61-99 in Fiechter A. (ed.) 1980b (q.v.).

WANG P. J. 1977 Regeneration of virus-free potato from tissue culture. pp. 386-391 in Barz W., Reinhard E. and Zenk M.H. (eds.) *Plant Tissue Culture and its Biotechnological Application*. Springer-Verlag, Berlin, Heidelberg, New York.

WAZIZUKA T. & YAMAGUCHI T. 1987 The induction of enlarged apical domes *in vitro* and multi-shoot formation from finger millet (*Eleusine coracana*). Ann. Bot. 60, 331-336.

WELANDER N.T. 1987 Propagation of *Syringa chinensis* cv. *Saugeana* by *in vitro* culture of nodal explants. J. Hort. Sci. 62, 89-96.

WERBROUCK S. P. O., DHUYVETTER H., PÉREZ R. M., TOPOONYANONT N. & DEBERGH P. 2000 Plant propagation *in vitro*: hormonal interactions. Acta Hortic. 560, 377-381.

WETHERELL D.F. 1969 Phytochrome in cultured wild carrot tissue. I. Synthesis. Plant Physiol. 44, 1734-1737.

WETHERELL D.F 1982 *Introduction to In Vitro Propagation*. Avery Publishing Group, Inc., Wayne, New Jersey.

WHITE P.R. 1933 Results of preliminary experiments on the culturing of isolated stem tips of *Stellaria media*. Protoplasma 19, 97-116.

WHITTIER D.P. & STEEVES T.A. 1962 Further studies on induced apogamy of ferns. Can. J. Bot. 40, 1525-1531.

WICKSON M. & THIMANN K.V. 1958 The antagonism of auxin and kinetin in apical dominance. Physiol. Plant. 11, 62-74.

YAE B.W., ZIMMERMAN R.H., FORDHAM I. & KO K.C. 1987 Influence of photoperiod, apical meristem and explant orientation on axillary shoot proliferation of apple cultivars *in vitro*. J. Am. Soc. Hortic. Sci. 112, 588-592.

YANG H.-J. & CLORE W.J. 1973 Rapid vegetative propagation of asparagus through lateral bud culture. HortScience 8, 141-143.

YANG H.-J. & CLORE W.J. 1974 Development of complete plantlets from moderately vigorous asparagus shoots of stock plants *in vitro*. HortScience 9, 138-140.

YANG Q.G., READ P.E., FELLMAN C.D. & HOSIER M.A. 1986 Effect of cytokinin, IBA and rooting regime on Chinese chestnut cultured *in vitro*. HortScience 21, 133-134.

ZEE S.-Y., & WU S.C. 1979 Embryogenesis in the petiole explants of Chinese celery. Z. Pflanzenphysiol. 93, 325-335.

ZEE S.-Y., WU S.C. & YUE S.B. 1979 Morphological and SDS-polyacrylamide gel electrophoretic studies of pro-embryoid formation in the petiole explants of Chinese celery. Z. Pflanzenphysiol. 95, 397-403.

ZILIS M.R. & ZWAGERMAN D. 1980 *In vitro* characteristics of nucellar and adventitious embryos in the genus *Hosta*. HortScience 15, 3.

ZIMMERMAN R.H., GRIESBACH R.J., HAMMERSCHLAG F.A. & LAWSON R.H. 2002 *Tissue Culture as Plant Production System*. Kluwer Academic Publishers, Dordrecht.

ZIV M. & HALEVY A.H. 1972 Organs and plantlets regeneration of gladiolus through tissue culture. Proc. Int. Hort. Cong. 18 (1970) 1, 211-212.

ZIV M., HALEVY H. & SHILO R. 1970 Organs and plantlets regeneration of gladiolus through tissue culture. Ann. Bot. 34, 671-676.

ZIV M., KAHAMY, S. & LILIEN-KIPNIS, H. 1994 Scaled-up proliferation and regeneration of Nerine in liquid cultures Part I. The induction and maintenance of proliferating meristematic clusters by paclobutrazol in bioreactors. Plant Cell Tissue Organ Cult. 39, 109-115

Chapter 3
The Components of Plant Tissue Culture Media I: Macro- and Micro-Nutrients

1. INORGANIC MEDIUM COMPONENTS

Plant tissues and organs are grown *in vitro* on artificial media, which supply the nutrients necessary for growth. The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of the culture medium used. For healthy and vigorous growth, intact plants need to take up from the soil:

- relatively large amounts of some inorganic elements (the so-called major plant nutrients): ions of nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg) and sulphur (S); and,
- small quantities of other elements (minor plant nutrients or trace elements): iron (Fe), nickel (Ni), chlorine (Cl), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo).

According to Epstein (1971), an element can be considered to be essential for plant growth if:

1. a plant fails to complete its life cycle without it;
2. its action is specific and cannot be replaced completely by any other element;
3. its effect on the organism is direct, not indirect on the environment;
4. it is a constituent of a molecule that is known to be essential.

The elements listed above are - together with carbon (C), oxygen (O) and hydrogen (H) - the 17 essential elements. Certain others, such as cobalt (Co), aluminium (Al), sodium (Na) and iodine (I), are essential or beneficial for some species but their widespread essentiality has still to be established.

The most commonly used medium is the formulation of Murashige and Skoog (1962). This medium was developed for optimal growth of tobacco callus and the development involved a large number of dose-response curves for the various essential minerals. Table 3.1 shows the composition of MS compared to the elementary composition of normal, well-growing plants. From this table, the relatively low levels of Ca, P and Mg in MS are evident. The most striking differences are the high levels of Cl and Mo and the low level of Cu. Each plant species has its own characteristic elementary composition which can be used to adapt the medium formulation. These media result often in a much improved growth (Rugini, 1984; El Badaoui *et al.,* 1996; Pullman *et al.,* 2003; Bouman and Tiekstra, 2005; Nas and Read, 2004; Gonçalves *et al.,* 2005). A major problem in changing the mineral composition of a medium is precipitation, which may often occur only after autoclaving because of the endothermic nature of the process.

Plant tissue culture media provide not only these inorganic nutrients, but usually a carbohydrate (sucrose is most common) to replace the carbon which the plant normally fixes from the atmosphere by photosynthesis. To improve growth, many media also include trace amounts of certain organic compounds, notably vitamins, and plant growth regulators.

In early media, 'undefined' components such as fruit juices, yeast extracts and protein hydrolysates, were frequently used in place of defined vitamins or amino acids, or even as further supplements. As it is important that a medium should be the same each time it is prepared, materials, which can vary in their composition are best avoided if at all possible, although improved results are sometimes obtained by their addition. Coconut milk, for instance, is still frequently used, and banana homogenate has been a popular addition to media for orchid culture.

Plant tissue culture media are therefore made up from solutions of the following components:

- macronutrients (always employed);
- micronutrients (nearly always employed but occasionally just one element, iron, has been used);
- sugar (nearly always added, but omitted for some specialised purposes);
- plant growth substances (nearly always added)
- vitamins (generally incorporated, although the actual number of compounds added, varies greatly);
- a solidifying agent (used when a semi-solid medium is required. Agar or a gellan gum are the most common choices).
- amino acids and other nitrogen supplements (usually omitted, but sometimes used with advantage);

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Table 3.1 A comparison between the average concentrations of elements in plant shoots (dry weight basis) considered sufficient for adequate growth [from Epstein (1972), content of Ni is according to Brown *et al.* (1987)] and in MS. The elements that show striking differences between MS and 'plants' are indicated. For Na, no data were found, but in glycophytes grown in 1 mM Na, the endogenous level is 10 – 1000 $mmol.kg^{-1}$ (Subbarao *et al.,* 2003)

	In tissue mmol kg^{-1}	**In MS mmol l^{-1}**	**In tissue mol%**	**In MS mol%**
N	1000	60	64.4	64.0
K	250	20	16.1	21.3
Ca	125	3	8.0	3.2
Mg	80	1.5	5.1	1.6
P	60	1.25	3.9	1.3
S	30	1.5	1.9	1.6
Cl	3	6	0.19	6.4
Fe	2	0.1	0.13	0.11
Mn	1	0.1	0.06	0.11
B	2	0.1	0.13	0.11
Zn	0.3	0.03	0.02	0.03
Cu	0.1	0.0001	0.0060	0.0001
Mo	0.001	0.001	0.0001	0.0011
Ni	0.001	0	0.0001	0.0000
Na		0.1	0.0000	0.1067

- undefined supplements such as coconut milk etc. (which, when used, contribute some of the five components above and also plant growth substances or regulants);
- buffers (have seldom been used, but the addition of organic acids or buffers could be beneficial in some circumstances).

Finally, it should be noted that minerals may also have a signalling role altering developmental patterns. This is most obvious in root architecture (Lopez-Bucio *et al.,* 2003) which is logical as roots have a principal function in ion uptake and the root system should be such that uptake is optimal. So growth and branching of roots should be affected by mineral concentrations in the soil. Ramage and Williams (2002) also argue that minerals appear to have an important role in the regulation of plant morphogenesis as opposed to just growth. Some reviews of whole plant mineral nutrition will be found in Grusak (2001), Leiffert *et al.,* (1995), Mengel and Kirkby (1982), Hewitt and Smith (1975) and Epstein (1971).

1.1. UPTAKE OF INORGANIC NUTRIENTS

Plants absorb the inorganic nutrients they require from soils almost entirely as ions. An ion is an atom, or a group of atoms, which has gained either a positive charge (a cation) or a negative charge (an anion). Inorganic nutrients are added to plant culture media as salts. In weak aqueous solutions, such as plant media, salts dissociate into cations and anions. Thus calcium, magnesium and potassium are absorbed by plant cells (normally those of the root) as the respective cations Ca^{2+}, Mg^{2+} and K^+; nitrogen is mainly absorbed in the form nitrate (the anion, NO_3^-) although uptake of ammonium (the cation, NH_4^+) may also occur, phosphorus as the phosphate ions HPO_4^{2-} and $H_2PO_4^-$; and sulphur as the sulphate ion SO_4^{2-}. In tissue culture, uptake is generally proportional to the medium concentration up to a concentration of twice MS (Williams, 1993). For specific elements this may be different. For example, Leiffert *et al,* (1995) found only a small increase in Zn uptake with increasing medium concentration indicating that the concentration of Zn in the cultured tissues was adequate, not requiring further uptake. Selective uptake also suggests active uptake.

Table 3.2 Content (mmol/kg) of elements in various agar brands.
[agar 1-7: Scholten and Pierik (1998); agar 8 and gelrite: Scherer *et al.*(1988)].
Na = not analysed, nd = not detected. It should be noted that some elements present in agar are not present in MS. This is particularly relevant for Ni which is an essential element

	Agar 1	Agar 2	Agar 3	Agar 4	Agar 5	Agar 6	Agar 7	Agar 8	gelrite
N	53	1	178	100	74	54	2	na	na
K	2	1	16	9	6	13	2	51	718
Ca	68	41	34	137	66	1	5	2.8	123
Mg	28	24	31	29	48	3	3	2.6	64
Na	202	56	552	330	427	634	114	52	296
P	1	18	1	5	1	40	1	42	68
S	184	78	232	296	204	262	66	184	6
Cl	47	33	220	113	197	95	12	na	na
Cu	0.015	0.034	0.018	0.024	0.004	0.016	nd	0.005	0.05
Mn	0.073	1.093	0.036	0.173	0.036	0.027	0.055	0.01	0.1
Fe	0.510	5.376	0.564	2.987	0.859	0.599	0.528	0.6	5
Al	0.352	12.444	1.333	4.944	0.352	0.963	0.685	0.3	6.8
Cr	0.040	0.098	0.029	0.026	0.054	0.025	0.009	0.002	0.01
Cd	0.013	0.069	0.008	0.025	0.015	nd	nd	0.0002	0.002
Zn	0.092	0.107	0.054	0.933	0.046	0.038	0.015	0.02	0.3
Sn	1.896	1.542	nd	3.572	1.862	nd	nd	0.003	0.003
Ni	0.037	0.045	nd	0.037	0.025	nd	0.007	0.005	0.004
B	na	na	na	na	na	na	na	2	0.13
Co	na	na	na	na	na	na	na	0.0005	1.0

Table 3.3 Increase of the content of Na, S and Cu relative to MS caused by adding agar (0.6%) or gelrite (0.2%) to the medium. Increases are shown as percentages. The proportional increase in other elements is maximally 20%

	Agar 1	Agar 2	Agar 3	Agar 4	Agar 5	Agar 6	Agar 7	Agar 8	gelrite
Na	1212	336	3312	1980	2562	3804	684	313	591
S	69	29	87	111	77	98	25	69	0.8
Cu	90	204	108	144	24	96	nd	28	91

In the whole plant, nutrients are either taken up passively, or through active mechanisms involving the expenditure of energy. Active uptake is generally less dependent on ionic concentration than passive uptake. Both systems are however influenced by the concentration of other elements, pH, temperature, and the biochemical or physiological status of the plant tissues. These factors can in turn be controlled by the solution presented to the roots, or they may dictate the ionic balance of an ideal solution. For example, Mg^{2+} competes with other cations for uptake. Under conditions of high K^+ or Ca^{2+} concentrations, Mg deficiency can result, and vice versa. Active uptake of phosphate falls off if the pH of the solution should become slightly alkaline when the $(H_2PO_4)^-$ ion becomes changed to $(HPO_4)^{2-}$. There is some evidence that ammonium is utilised more readily than nitrate at low temperatures and that uptake may be enhanced by high carbohydrate levels within plant cells. Calcium is not absorbed efficiently and concentrations within plant tissues tend to be proportional to those in the soil. Plants are comparatively insensitive to sulphate ions, but high concentrations of dissolved phosphate can depress growth, probably through competitively reducing the uptake of the minor elements Zn, Fe and Cu. Although the biochemistry and physiology of nutrient uptake in tissue cultures may be similar, it is unlikely to be identical.

In vivo, plants take up mineral ions with their roots. No studies have been made on how uptake of nutrients occurs in shoot cultures. For IAA, it has been shown that most uptake is *via* the cut surface and that only a small fraction is taken up via the

epidermis (Guan and De Klerk, 2000). The same likely holds for minerals. It should be noted, though, that in tissue culture the stomata are always open in the portion of the explant exposed to the gaseous phase (De Klerk and Wijnhoven, 2005) and the same may apply for tissues that are exposed to semi-solid or liquid medium. Uptake via the stomata is well possible.

Once taken up, transport within the plant occurs in the mass flow via the xylem. In *in vivo* plants there are two mechanisms for driving the water flow, root pressure and water potential gradient between at one end the soil and at the other end the atmosphere. Under normal conditions, the latter is the far more important, but water flow resulting from root pressure is in itself sufficient for long-distance mineral supply (Tanner and Beevers, 2001). Plants without roots are often cultured *in vitro* where the atmosphere is very humid, and the flow driven by a difference in water potential consequently reduced. In spite of this, in tissue culture there still seems to be sufficient water flow (Beruto *et al.,* 1999) which may be favoured by the stomata being continuously open (De Klerk and Wijnhoven, 2005). There are no indications that the structure of the xylem is altered in such a way as to reduce transport of ions.

When explants are first placed onto a nutrient medium, there may be an initial leakage of ions from damaged cells, especially metallic cations (Na^+, Ca^{2+}, K^+, Mg^{2+}) for the first 1-2 days, so that the concentration in the plant tissues actually decreases (Chaillou and Chaussat, 1986). Cells then commence active absorption and the internal concentration slowly rises. Phosphate and nitrogen (particularly ammonium) are absorbed more rapidly than other ions. In liquid medium, almost all phosphorus and ammonium are taken up in the first two weeks of culture (e.g. by 5 microshoots of *Dahlia* in 50 ml stationary liquid medium; G. de Klerk, unpublished results). After uptake, phophorus is massively redistributed to tissues that are formed after the initial two weeks. Nitrate in a medium very similar to that of Wood and Braun (1961) B medium, was reduced by *Catharanthus roseus* suspensions from 24 mM to 5 mM in 15 days, while Na^+, K^+, and SO_4^{2-}, fell to only just over half the original concentrations in the same time (MacCarthy *et al.,* 1980). Carnation shoot cultures were found to use 31-75% Mg^{2+}, and 29-41% Ca^{2+} in MS medium during a 4 week period (Dencso, 1987).

1.2. UNINTENDED ALTERATIONS

Nutrients, and especially micronutrients, may also be added via impurities, and especially via agar. Such impurities may well be beneficial. This is particularly true of Ni, which has recently been shown to be an essential element (Gerendás *et al.*, 1999) but was not known to be when most medium formulations were established. This element is usually not included in the inorganic constituents but can be provided by impurities. Tables 3.2 and 3.3 show impurities of various agar brands and their relative contribution to MS. Agar provides a large addition of sodium but levels of sulphur and copper are also significantly increased. Increases in the other elements in MS, are less than 20 %. Gelrite contains fewer organic impurities but inorganic ones occur at high concentrations (Table 3.2). It should be noted that the data in Table 3.2 are from determinations done more than 15 years ago and that the production process of gelrite has been improved ever since. Gelrite is being used in medicines as an ophthalmic vehicle. Furthermore, minerals are absorbed to a significant percentage by agar (Scholten and Pierik, 1998 Leiffert *et al.*, 1995) and by activated charcoal (Van Winkle *et al.*, 2003) but whether this has a significant effect has not been examined.

2. MACRONUTRIENTS

2.1. NITROGEN

2.1.1. Forms of nitrogen

Nitrogen is essential to plant life. It is a constituent of both proteins and nucleic acids and also occurs in chlorophyll. Most animals cannot assimilate inorganic nitrogen or synthesize many of the amino acids unless assisted by bacteria (e.g. in the rumen of cattle). Nitrogen is available in the atmosphere as N_2 but only legumes have the capacity to utilize this nitrogen using *Rhizobium* bacteria in the root nodules. In most plants, nitrate (NO_3^-) is the sole source of nitrogen. After uptake, NO_3^- is reduced to NH_4^+ prior to incorporation into organic molecules. (The removal of oxygen from a chemical compound and its replacement by hydrogen, is termed reduction.) The relevance of nitrogen is illustrated by the vast amounts of nitrogen reserves in seeds (as storage proteins).

Both growth and morphogenesis in tissue cultures are markedly influenced by the availability of nitrogen and the form in which it is presented. Compared to the nitrate ion, NO_3^- (which is a highly

oxidized form of nitrogen), the ammonium ion, NH_4^+, is the most highly ‘reduced’ form. Plants utilise reduced nitrogen for their metabolism and internally, nitrogen exists almost entirely in the reduced form. As a source of reduced nitrogen, plant cultures are especially able to use primary amines:

R-NH_2 and amides: R-CO-NH-R- (where R and R- are functional groups)

The primary amines which are most commonly employed in culture media are ammonia (NH_4^+) and, occasionally, amino acids.

Amides are less commonly added to culture media: those which can be used by plants are particularly

NH_2-CO-NH_2 (urea)

and ureides, which include allantoin and allantoic acid (Kirby, 1982) (Fig. 3.1). Allantoin or allantoic acid are sometimes more efficient nitrogen sources than urea (Lea *et al.,* 1979).

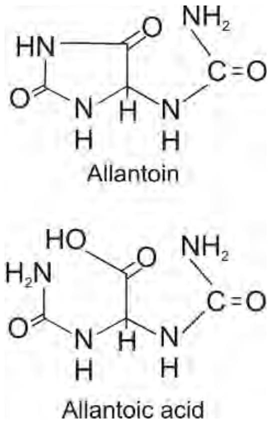

Fig. 3.1 The structures of allantoin and allantoic acid.

Most media contain more nitrate than ammonium ions, but as plant tissue culture media are usually not deliberately buffered, the adopted concentrations of ammonium and nitrate ions have probably been more due to practical pH control, than to the requirement of the plant tissues for one form of nitrogen or another (see chapter 4). Uptake of nitrate only takes place effectively in an acid pH, but is accompanied by extrusion of anions from the plant, leading to the medium gradually becoming less acid. By contrast, uptake of ammonium results in the cells excreting protons (H^+) into the medium, making it more acid. The exchange of ions preserves the charge balance of the tissues and may also assist in the disposal of an excess of protons or hydroxyl (OH^-) ions generated during metabolism (Raven, 1986). Uptake of nitrogen by cell suspension cultures of *Nicotiana tabacum* is an active (energy-dependent) process (Heimer and Filner, 1971) and is dependent on a supply of oxygen (Buwalda and Greenway, 1989).

Plant culture media are usually started at pH 5.4-5.8. However, in one containing both nitrate and ammonium ions, a rapid uptake of ammonium into plant tissue causes the pH to fall to ca. 4.2-4.6. As this happens, further ammonium uptake is inhibited, but uptake of nitrate ion is stimulated, causing the pH to rise again. In unbuffered media, efficient nitrogen uptake can therefore depend on the presence of both ions. Unless otherwise stated, comments in this section on the roles of nitrate and ammonium refer to observations on unbuffered media.

There is generally a close correlation in tissue cultures between uptake of nitrogen, cell growth and the conversion of nitrogen to organic materials. A readily available supply of nitrogen seems to be important to maintain cultured cells in an undifferentiated state. The depletion of nitrogen in batch cultures, triggers an increase in the metabolism of some nitrogen-free compounds based on phenylpropanes (such as lignin), which are associated with the differentiation of secondarily-thickened cells (Hahlbrock, 1974). However, the growth in culture of differentiated cotton fibres composed largely of cellulose, is nitrate-dependent; the presence of some reduced nitrogen in the culture medium decreased the proportion of cultured embryos which produced fibres, and particularly in the absence of boron, promoted the cells of the embryos to revert to callus formation (Birnbaum *et al.,* 1974).

2.1.2. Nitrate ions (NO_3^-)

Nitrate ions are an important source of nitrogen for most plant cultures, and nearly all published media provide the majority of their available nitrogen in this form. However, once within the cell, nitrate has to be reduced to ammonium before being utilised biosynthetically. Why not simply supply nitrogen as NH_4^+ and avoid the use of NO_3^- altogether? The reason lies in the latent toxicity of the ammonium ion in high concentration, and in the need to control the pH of the medium.

Conversion of nitrate to ammonium is brought about firstly by one, or possibly two, nitrate reductase enzymes, which reduce NO_3^- to nitrite (NO_2^-). One nitrate reductase enzyme is thought to be located in the cytoplasm, while the second may be bound to membranes (Nato *et al.,* 1990). The NO_2^- produced by the action of nitrate reductase is reduced to NH_4^+ by a nitrite reductase enzyme located in plastids (Fig. 3.2). Reduction of nitrate to ammonia requires the

cell to expend energy. The ammonium ions produced are incorporated into amino acids and other nitrogen-containing compounds. Nitrate and nitrite reductase enzymes are substrate induced, and their activity is regulated directly by the level of nitrate-nitrite ions within cultured cells (Chroboczek-Kelker and Filner, 1971; Hahlbrock, 1974), but also apparently by the products of the assimilation of reduced nitrogen (see below).

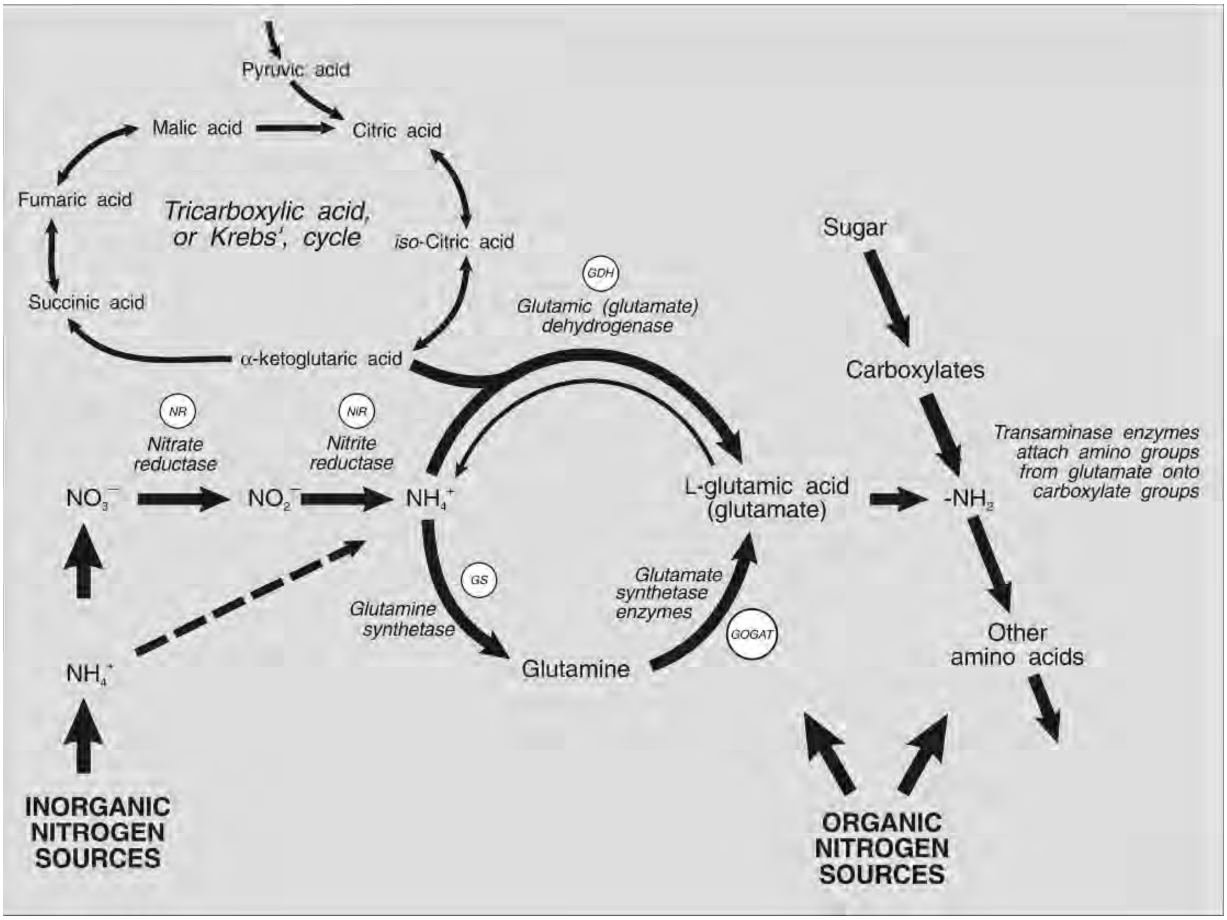

Fig. 3.2 The metabolism of nitrate and ammonium ions.

Unlike the ammonium ion, nitrate is not toxic and, in many plants, much is transported to the shoots for assimilation. On the other hand, the nitrite ion can become toxic should it accumulate within plant tissues or in the medium, for example when growth conditions are not favourable to high nitrite reductase activity and when nitrate is the only nitrogen source (Jordan and Fletcher, 1979; Grimes and Hodges, 1990). In *Pinus pinaster*, nitrate reductase is induced by the presence of KNO_3, and plants regenerated *in vitro* exhibit an ability to reduce nitrate similar to that of seedlings (Faye *et al.,* 1986).

For most types of culture, the nitrate ion needs to be presented together with a reduced form of nitrogen (usually the NH_4^+ ion), and tissues may fail to grow on a medium with NO_3^- as the only nitrogen source (Hunault, 1985).

2.1.3. Reduced nitrogen

In the natural environment and under most cropping conditions, plant roots usually encounter little reduced nitrogen, because bacteria rapidly oxidize available sources (Hiatt, 1978). An exception is forest soils in mountainous regions of the northern hemisphere, where nitrates are not usually available (Durzan, 1976). If NH_4^+, and other reduced nitrogen compounds are available, (and this is particularly the case in the aseptic *in vitro* environment), they can be taken up and effectively utilized by plants. In fact the uptake of reduced nitrogen gives a plant an

ergonomic advantage because the conversion of nitrate to ammonium ions (an energy-requiring process) is not necessary. The free ammonium ion can cause toxicity, which, at least in whole plants, can lead to an increase in ethylene evolution (Barker and Corey, 1987; Corey and Barker, 1987). Shoots grown on an unbuffered medium containing a high proportion of ammonium ions may become stunted or hyperhydric. These effects can sometimes be reversed by transfer to a medium containing a high proportion of NO_3^- or to one where NO_3^- is the only N source (Mott *et al.,* 1985). Hyperhydricity is the *in vitro* formation of abnormal organs, which are brittle and have a water-soaked appearance.

Growth of plant cultures may also be impaired in media containing high concentrations of NH_4^+ even when high concentrations of NO_3^- are present at the same time. Growth inhibition may not only be due to depressed pH (Mott *et al.,* 1985), but may reflect a toxicity induced by the accumulation of excess ammonium ions. In normal circumstances the toxic effect of ammonium is avoided by conversion of the ion into amino acids. There are two routes by which this takes place (Fig. 3.2), the most important of which, under normal circumstances, is that by which L-glutamic acid is produced from glutamine through the action of glutamine synthetase (GS) and glutamate synthetase (GOGAT) enzymes. Compounds, which block the action of GS can be used as herbicides (De Greef *et al.,* 1989). The reaction of α-ketoglutaric acid with NH_4^+ is usually less important, but seems to have increased significance when there is an excess of ammonium ions (Furuhashi and Takahashi, 1982). Detoxification and ammonium assimilation may then be limited by the availabilty of α-ketoglutaric acid, but this may be increased *in vitro* by adding to the medium one or more acids which are Krebs' (tri-carboxylic acid) cycle intermediates. Their addition can stimulate growth of some cultures on media containing high levels of NH_4^+ (Gamborg, 1970).

In comparison with media having only nitrate as the nitrogen source, the presence of the ammonium ion in media usually leads to rapid amino acid and protein synthesis, and this takes place at the expense of the synthesis of carbohydrate compounds. This diversion of cellular metabolism can be disadvantageous in some shoot cultures, and can contribute towards the formation of hyperhydric shoots. Hyperhydricity no longer occurs when NH_4^+ is eliminated from the medium or greatly reduced. It is possible that adding an organic acid to the medium might also alleviate the symptoms on some plants.

A supply of reduced nitrogen in addition to nitrate, appears to be beneficial for at least two processes involved with cell division:

- the formation of a cell wall. Without a complete cell wall, protoplasts require a factor capable of inducing wall formation. Freshly-isolated protoplasts may contain sufficient of this substance to promote wall formation for just a few divisions. The wall-forming factor is only effective when NH_4^+ is present in the medium (Meyer and Abel, 1975a,b): glutamine does not substitute for NH_4^+:
- the activity of growth regulators. (see below)

There are several reports in the literature that, with constant amounts of NO_3^-, ammonium sulphate has not provided such a good source of NH_4^+ as ammonium nitrate or ammonium chloride (De Jong *et al.,* 1974; Steward and Hsu, 1977; Singh, 1978; Kamada and Harada, 1979). Possibly the reason is that a medium containing ammonium sulphate has a greater tendency to become acid (Harris, 1956), than one containing less sulphate ions. This would result if the presence of sulphate ions accelerated the uptake of NH_4^+, or slowed the uptake of NO_3^-. Ammonium sulphate has been used as the only source of the ammonium ion in some media used for the culture of legumes, including B5 (Gamborg *et al.,* 1968).

2.1.4. Ammonium as the sole nitrogen source

pH adjustment. If plant tissues are presented with a medium containing only NH_4^+ nitrogen, the pH falls steadily as the ion is taken up (for example, a decrease of 0.9 pH units in 15 days in *Asparagus* callus – Hunault (1985) or 0.7 pH units with potato shoots – Avila *et al.,* (1998). Growth and morphogenesis is possible in suspension cultures containing only NH_4^+ ions, providing the pH of the medium is frequently adjusted by the addition of a base (Martin *et al.,* 1977), or the medium is buffered (see below). In wild carrot, the induction of embryogenesis required the medium to be adjusted to pH 5.4 at 8 hourly intervals (Dougall and Verma, 1978). Without adjustment, the pH of media containing only NH_4^+ falls rapidly to a point where cells cannot grow (Dougall, 1981).

Buffering. Ammonium can also serve as the only nitrogen source when the medium is buffered (see the section on pH, below). Tobacco cells could be grown on a medium containing NH_4^+ nitrogen if the organic acid ion, succinate, was added to the medium. Gamborg and Shyluk (1970) found that cultured cells

could be grown without frequent pH adjustment on a medium containing only NH_4^+ nitrogen, when a carboxylic acid was present. The organic acids appeared to minimize the acidification of the medium through NH_4^+ uptake. Similarly *Asparagus* internode callus grew just as well on NH_4^+ as the only nitrogen source as on a medium containing both NH_4^+ and NO_3^-, but only when organic acids (such as citrate, or malate) or MES buffer were added to the medium. When media were buffered with MES, the best callus growth occurred when the pH was 5.5 (Hunault, 1985).

The additional effect of organic acids. Although Krebs' cycle organic acids can act as buffers, they may also act as substrates for amino acid synthesis from NH_4^+. To be assimilated into amino acids via the GDH enzyme, the ammonium ion must react with α-ketoglutaric acid, which is produced by the Krebs' cycle (Fig 3.2). Its availability may govern the rate at which ammonium can be metabolised by this route. The rate of assimilation might be expected to be improved by supplying the plant with α-ketoglutarate directly, or by supplying acids which are intermediates in the Krebs' cycle (citrate, iso-citrate, succinate, fumarate or malate), for then the natural production of α-ketoglutarate should increase (Gamborg, 1970).

This hypothesis was confirmed by Behrend and Mateles (1976) who concluded that succinate, or other Krebs' cycle acids, acted mainly as a nutrient, replacing α-ketoglutarate as it was withdrawn from the cycle during NH_4^+ metabolism and amino acid synthesis. Depletion of α-ketoglutarate causes the cycle to cease unless it, or another intermediate, is replaced. The optimum molar ratio of NH_4^+ to succinate, was 1.5 (e.g. 10 mM NH_4^+: 15 mM succinate). Chaleff (1983) thought that the growth of rice callus on Chaleff (*loc. cit.*) R3 (NH_4) medium, containing 34 mM of only ammonium nitrogen, [Chaleff (1983) R3 NH_4 medium] was enabled by the presence of 20 mM succinate or a-ketoglutarate, partly by the buffering capacity of the acids, and partly by their metabolism within the plant, where they may serve as substrate for amino acid synthesis. Similar conclusions have been reached by other workers (e.g. Fukunaga *et al.,* (1978); Dougall and Weyrauch (1980); Hunault (1985); Molnar (1988b), who have found that compounds such as ammonium malate and ammonium citrate are effective nitrogen sources.

Orange juice promotes the growth of *Citrus* callus. Einset (1978) thought that this was not due to the effect of citric acid, but Erner and Reuveni (1981) showed that citric acid, particularly at concentrations above the 5.2 mM found in the juice used by Einset, does indeed promote the growth of *Citrus* callus; it had a more pronounced effect than other Krebs' cycle acids, perhaps due to the distinctive biochemistry of the genus.

Organic acids not only enhance ammonium assimilation when NH_4^+ provides the only source of nitrogen, but may sometimes also do so when nitrate ions are in attendance. The weight of rice anther callus was increased on Chaleff (1983) R3 medium, if 20 mM succinate [Chaleff (1983) R3 Succ. medium] or α-ketoglutarate was added (Chaleff, 1983). Similarly the rate of growth of *Brassica nigra* suspensions on MS medium, was improved either by adding amino acids, or 15 mM succinate. An equivalent improvement (apparently due entirely to buffering) only occurred through adding 300 mM MES buffer (Molnar, 1988b). However, the presence of organic acids may be detrimental to morphogenesis. In Chaleff's experiment, the presence of succinate in R3 medium markedly decreased the frequency of anther callus formation.

Photosynthesis. Although plants grown on nutrient solutions containing only NH_4^+ nitrogen have been found to possess abnormally high levels of PEP enzyme (Arnozis *et al.,* 1988) (the enzyme facilitating CO_2 fixation in photosynthesis), media containing high levels of NH_4^+ tend to inhibit chlorophyll synthesis (Yoshida and Kohno, 1982) and photosynthesis.

2.1.5. Urea

Plants are able to absorb urea, but like the ammonium ion, it is not a substance that is normally available in soils in the natural environment. It is however produced as a by-product of nitrogen metabolism; small quantities are found in many higher plants, which are able to utilise urea as a source of nitrogen, providing it is first converted to ammonium ions by the enzyme urease. In legumes and potato, urease requires the microelement nickel for activity (see below). In conifers, the epidermal cells of cotyledons and cotyledons are capable of urease induction and ammonium ion formation (Durzan, 1987).

Urea can be used as the sole nitrogen source for cultures, but growth is less rapid than when ammonium and nitrate ions are supplied (Kirkby *et al.,* 1987); urease enzyme increases after cultures have been maintained for several passages on a urea-based medium (King, 1977; Skokut and Filner,

1980). Although the metabolism of urea, like that of other reduced nitrogen compounds, causes the production of excess hydrogen ions, less are predicted to be secreted into the medium than during the utilisation of NH_4^+ (Raven, 1986), so that urea is less suitable than ammonium to balance the pH of media containing NO_3^-. Nitrate ions are utilized in preference to urea when both nitrogen sources are available (King, 1977). Urea is able to serve as a reduced nitrogen source during embryogenesis (Durzan, 1987), but has been used in relatively few culture media, and of these, none has been widely adopted (George *et al.,* 1987).

2.1.6. Media with nitrate and ammonium ions

Most intact plants, tissues and organs take up nitrogen more effectively, and grow more rapidly, on nutrient solutions containing both nitrate and ammonium ions, than they do on solutions containing just one of these sources. Although in most media, reduced nitrogen is present in lower concentration than nitrate, some morphogenic events depend on its presence, and it can be used in plant cultures in a regulatory role. Adventitious organs may also develop abnormally if NH_4^+ is missing (Drew, 1987).

Possible explanations, which have been put forward for the regulatory effect of NH_4^+ are:

- that the reduction and assimilation of NO_3^- is assisted by the presence of of NH_4^+ or the products of its assimilation (Bayley *et al.,* 1972a,b; Mohanty and Fletcher, 1978; 1980). When grown on a medium containing a small amount of NH_4^+ nitrogen in addition to nitrate, suspension cultured cells of 'Paul's Scarlet' rose accumulated twice as much protein as when grown on a medium containing only nitrate, even though ammonium finally accounted for only 10% of the total protein nitrogen (Mohanty and Fletcher, 1980). Dougall (1977) considered this to be an oversimplified interpretation, moreover nitrate reductase activity is effectively increased by the presence of NO_3^- (Müller and Mendel, 1982) and in some plants, a high concentration of NH_4^+ inhibits nitrate reductase activity (see below).
- that ammonium ions effectively buffer plant nutrient media in the presence of nitrate and so enhance nitrate uptake (see the section on pH).

Cultures of some plants are capable of growing with only NO_3^- nitrogen (e.g. cell cultures of *Reseda luteoli*, soybean, wheat, flax and horse radish – Gamborg (1970); callus of *Medicago sativa* - Walker and Sato (1981), although yields are generally better when the medium is supplemented with NH_4^+. Craven *et al.,* (1972) with carrot, and Mohanty and Fletcher (1978) with *Rosa* 'Paul's Scarlet', found that the presence of NH_4^+ was particularly important during the first few days of a suspension culture. After that cells increase in cell number and dry weight more rapidly on NO_3^- nitrogen alone.

The response of plant cultures to nitrate and ammonium ions depends to a large extent on the enzymes shown in Fig 3.2, and the manner in which their activities are increased or inhibited in different tissues by the presence of the ions. These factors vary according to the degree of differentiation of the tissue (Suzuki and Nato, 1982), its physiological age, and its genotype. For example, the high level of NH_4^+ in MS medium inhibited the activity of glutamate synthetase enzyme in soybean suspension cultures (Gamborg and Shyluk, 1970), while in *N. tabacum*, a peak of glutamate dehydrogenase (GDH) appeared to exist at 10 mM NH_4^+ (Lazar and Collins, 1981). The activity of GDH and NADH-dependent GOGAT developed rapidly in cultured tobacco cells, while nitrate reductase and ferridoxin-dependent GOGAT activity increased more slowly during growth. By contrast, in sunflower cultures, the specific activity of GDH and ferridoxin-dependent GOGAT only reached a maximum at the end of growth, and the presence of 15 mM NH_4^+ inhibited the activity of nitrate reductase, indicating that the cells were entirely dependent on the reduced nitrogen in the medium (Lenee and Chupeau, 1989).

In consequence of such variation, the relative concentrations of ammonium and nitrate in media may need to be altered for different cultures.

2.1.7. The correct balance of ions

When trying to find media formulations suitable for different plant species and different kinds of cultures, two important factors to be considered are:

- the total concentration of nitrogen in the medium;
- the ratio of nitrate to ammomium ions.

There is a high proportion of NH_4^+ nitrogen in MS medium [ratio of NO_3^- to NH_4^+, 66:34] and the quantity of total nitrogen is much higher than that in the majority of other media. For some cultures, the total amount of nitrogen is too high and the balance between the two forms in the medium is not optimal.

It is noticeable that in several media used for legume culture, there is a greater proportion of NO_3^- to NH_4^+ ions than in MS medium. Evans *et al.,* (1976) found that soybean leaf callus grew more rapidly and formed more adventitious roots when the ammonium nitrate in MS medium was replaced with

500 mg/l ammonium sulphate. A reduction in the ammonium level of their medium for the callus culture of red clover, was also found to be necessary by Phillips and Collins (1979, 1980) to obtain optimum growth rates of suspension cultured cells. A similar adjustment can be beneficial in other genera. Eriksson (1965) was able to enhance the growth rate of cell cultures of *Haplopappus gracilis* when he reduced the ammonium nitrate concentration to 75% of that in MS medium, and doubled the potassium dihydrogen phosphate level.

Table 3.4 lists examples of where changes to the nitrogen content of MS medium resulted in improved *in vitro* growth or morphogenesis. It will be seen that the balance between NO_3^- and NH_4^+ in these different experiments has varied widely. This implies that the ratio between the two ions either needs to be specifically adjusted for each plant species, or that the total nitrogen content of the medium is the most important determinant of growth or morphogenesis. Only occasionally is an even higher concentration of nitrate than that in MS medium beneficial. Trolinder and Goodin (1988) found that the best growth of globular somatic embryos of *Gossypium* was on MS medium with an extra 1.9 g/l KNO_3.

There are reports that adjustments to the nitrogen content and ratio of NO_3^- to NH_4^+, can be advantageous in media containing low concentrations of salts (Table 3.5). Biedermann (1987) found that even quite small adjustments could be made advantageously to the NO_3^- content of a low salt medium [that of Biedermann (1987)] for the shoot culture of different *Magnolia* species and hybrids, but too great a proportion of NO_3^- was toxic.

2.1.8. The nitrate-ammonium ratio for various purposes

Root growth. Root growth is often depressed by NH_4^+ and promoted by NO_3^-. Media for isolated root culture contain no NH_4^+, or very little. Although roots are able to take up nitrate ions from solutions, which become progressively more alkaline as assimilation proceeds, the same may not be true of cells, tissues and organs *in vitro*.

Shoot cultures. Media containing only nitrate nitrogen are used for the shoot culture of some plants, for example strawberry (Boxus, 1974), which can be cultured with 10.9 mM NO_3^- alone; supplementing the medium with 6 mM NH_4^+ causes phytotoxicity (Damiano, 1980). However, shoot cultures of strawberry grown without NH_4^+ can become chlorotic: adding a small amount of NH_4NO_3 (or another source of reduced nitrogen) to the medium at the last proliferation stage, or to the rooting medium, may then give more fully developed plants with green leaves (Zimmerman, 1981; Piagnani and Eccher, 1988). On some occasions it is necessary to eliminate or reduce NH_4^+ from the medium for shoot cultures to prevent hyperhydricity.

2.1.9. Nitrogen supply and morphogenesis

Morphogenesis is influenced by the total amount of nitrogen provided in the medium and, for most purposes, a supply of both reduced nitrogen and nitrate seems to be necessary. The requirement for both forms of nitrogen in a particular plant species can only be determined by a carefully controlled experiment: simply leaving out one component of a normal medium gives an incomplete picture. For example, cotyledons of lettuce failed to initiate buds when NH_4NO_3 was omitted from Miller (1961) salts and instead formed masses of callus (Doerschug and Miller, 1967): was this result due to the elimination of NH_4^+, or to reducing the total nitrogen content of the medium to one third of its original value?

Importance of the nitrate/ammonium balance. The importance of the relative proportions of NO_3^- and NH_4^+ has been demonstrated during indirect morphogenesis and the growth of regenerated plants. Grimes and Hodges (1990) found that although the initial cellular events which led to plant regeneration from embryo callus of indica rice, were supported in media in which total nitrogen ranged from 25 to 45 mM and the NO_3^- to NH_4^+ ratio varied from 50:50 to 85:15 (Table 3.5), differentiation and growth were affected by very small alterations to the NO_3^- to NH_4^+ ratio. Changing it from 80:20 (N6) medium, to 75:20, brought about a 3-fold increase in plant height and root growth, whereas lowering it below 75:25, resulted in short shoots with thick roots. Atypical growth, resulting from an unsuitable balance of nitrate and ammonium, has also been noted in other plants. It gave rise to abnormal leaves in *Adiantum capillus-veneris* (Pais and Casal, 1987); the absence of ammonium in the medium caused newly initiated roots of *Carica papaya* to be abnormally thickened, and to have few lateral branches (Drew, 1987). Shoot cultures may survive low temperature storage more effectively when maintained on a medium containing less NH_4NO_3 than in MS medium (Moriguchi and Yamaki, 1989).

By comparing different strengths of Heller (1953; 1955) and MS media, and varying the NH_4NO_3 and $NaNO_3$ levels in both, David (1972) was led to the conclusion that the principal ingredient in MS favouring differentiation in Maritime pine explants is

NH_4NO_3. However, in embryonic explants of *Pinus strobus*, adventitious shoot formation was better on Schenk and Hildebrandt (1972) medium than on MS (which induced more callus formation). The difference in the ammonium level of the two media was mainly responsible (Flinn and Webb, 1986).

Table 3.4 Examples of adjustments of the nitrogen content of MS medium, which resulted in improved growth or morphogenesis. In each case, only NO_3^- and NH_4^+ were changed, the rest of the medium being the same except for K^+ and Cl^-

Plant species	Type of culture	Results	NO_3^- (mM)	NH_4^+ (mM)	Ratio of NO_3^- to NH_4^+	Total N (mM)	Reference
Nicotiana tabacum	Callus	Optimum callus growth	39.4	20.61	1.91	60.01	Murashige and Skoog (1962)
Nicotiana tabacum	Callus	Equal callus growth and shoot formation	40.0	20.0	2.00	60.0	Behki and Lesley (1980)
			48.0	12.0	4.00	60.0	
			52.0	8.0	6.50	60.0	
	Callus	Callus growth and root formation	24.0	12.0	2.00	36.0	
Nicotiana tabacum	Shoot regeneration from leaf disks	Optimal number of shoots	40	20	2.3	60	Ramage and Williams (2002)
Dioscorea spp.	Callus	Callus growth and adventitious plantlets	6.25	6.25	1.00	15.0	Asokan *et al.*, (1983)
Diospyros kaki	Shoot	Shoot proliferation	19.7	20.61	0.96	40.31	Sugiura *et al.*, (1986)
Rubus ideaus	Shoot	Shoot proliferation	19.7	10.30	1.91	30.0	Welander (1987)
Prunus avium	Shoot	Shoot proliferation	29.09	10.3	2.82	39.40	Righetti *et al.*, (1988)
Castanea sativa and *Castanea* hybrids	Shoot	Shoot proliferation	18.00	3.00	6.00	21.0	Piagnani and Eccher (1988)
Peltophorum pterocarpum	Anther	Callus formation and shoot regeneration	34.7	9.99	3.47	44.6	Lakshmana Rao and De (1987)
Euphorbia esula	Suspension	Plant regeneration	18.00	8.00	2.25	27	Davis *et al.*, (1988)
Haplopappus gracillis	Suspension	Improved growth rate	33.78	14.99	2.25	8.77	Eriksson (1965)
Oryza sativa	Protoplast	Cell division	18.79	1.01	18.53	19.80	Yamada *et al.*, (1986)
Allium sativum	Shoot	Shoot proliferation	35.0	8.0	4.4	43.0	Luciani *et al.*, (2001)
Solanum tuberosum	Shoot	Shoot proliferation	41.3	17.7	2.3	59.0	Avila *et al.*, (1998)

Table 3.5 Examples of beneficial and harmful adjustments to the total nitrogen and the NO_3^- and NH_4^+ in low salt media

Basic Medium in which N modified	Plant species or variety	Type of culture and results	NO_3^- (mM)	NH_4^+ (mM)	Ratio of NO_3^- to NH_4^+	Total N (mM)	Reference
Biederman (1987)			**8.62**	**6.25**	**58:42(1.38)**	**14.87**	
	Magnolia stellata	Shoot culture: maximum proliferation	10.99	6.25	1.76	17.24	Biederman (1987)
	Magnolia 'Elizabeth'	Shoot culture: maximum proliferation	8.62-10.99	6.25	1.38 – 1.76	14.87 – 17.24	
	Magnolia 'Yellow Bird' and '#149'	Shoot culture: maximum proliferation	7.43	6.25	1.19	13.68	
	Magnolia (all vars.)	Shoot culture: death of all cultures	25.04	6.25	4.01	31.29	
Chu *et al.*, (1975) N6			**28.00**	**7.00**	**80:20(4.00)**	**35.00**	
	Oryza sativa	Callus induction from immature embryos	28.00	7.00	4.00	35.00	Grimes and Hodges (1990)
		Plant regeneration after callus induction with 2,4-D	12.5–38.25	22.5-3.75	1.0 - 5.66	25.00-45.00	
		Optimum number of plants per zygotic embryo	18.75	6.25	3.00	25.00	
		Poor regeneration	100 <17.5	0 >17.5	∞ <1.0	35.00	
		Optimum plantlet growth	26.25	8.75	8.75	35.00	

Morphogenesis influenced by total available nitrogen. Others have found that the total nitrogen content of culture media influences morphogenesis more than the relative ammonium concentration. Results of Margara and Leydecker (1978) indicated that adventitious shoot formation from rapeseed callus was optimal in media containing 30-45mM total nitrogen. The percentage of explants forming shoots was reduced on media containing smaller or greater amounts (e.g. on MS medium). Increasing the ratio of NH_4^+ to total N in media, from 0.20 to 0.33 was also detrimental. Similarly, Gertsson (1988) found that a small number of adventitious shoots was obtained on petiole segments of *Senecio hybridus* when the total nitrogen in MS medium was increased to 75 mM, but that an increased number of shoots was produced when the total nitrogen was reduced to 30 mM (while keeping the same ratio of NO_3^- to NH_4^+). Shoot production was more than doubled if, at the same time as the total N was reduced, the potassium ion concentration was fixed at 15 mM, instead of 20 mM.

The total amount of nitrogen in a medium was shown by Roest and Bokelmann (1975) to affect the number of adventitious shoots formed directly on *Chrysanthemum* pedicels. The combined amount of KNO_3 + NH_4NO_3 in MS medium (60 mM), was adjusted as is shown in Fig. 3.3 while the ratio of NO_3^- to NH_4^+ (66:34) was unchanged. From 30-120 mM total nitrogen was optimal. However there was clearly a strong effect of genotype, because the cultivar `Bravo' was much more sensitive to increased nitrogen than 'Super Yellow'.

Nitrogen x sugar interaction. The enhancement of morphogenesis caused by high nitrogen levels may not be apparent unless there is an adequate sucrose concentration in the medium (Margara and Rancillac, 1966; Gamborg *et al.,* 1974). In *Dendrobium*, the uptake of NO_3^- is slower than that of NH_4^+. Uptake is dependent on the nature and concentration of the sugar in the medium, being slower in the presence of fructose than when sucrose or glucose are supplied (Hew *et al.,* 1988). The rate of growth of *Rosa* 'Paul's Scarlet' suspensions was influenced by the ratio of NO_3^- to sucrose in the medium. A high ratio favoured the accumulation of reduced nitrogen, but not the most rapid rate of cell growth (Fletcher, 1980).

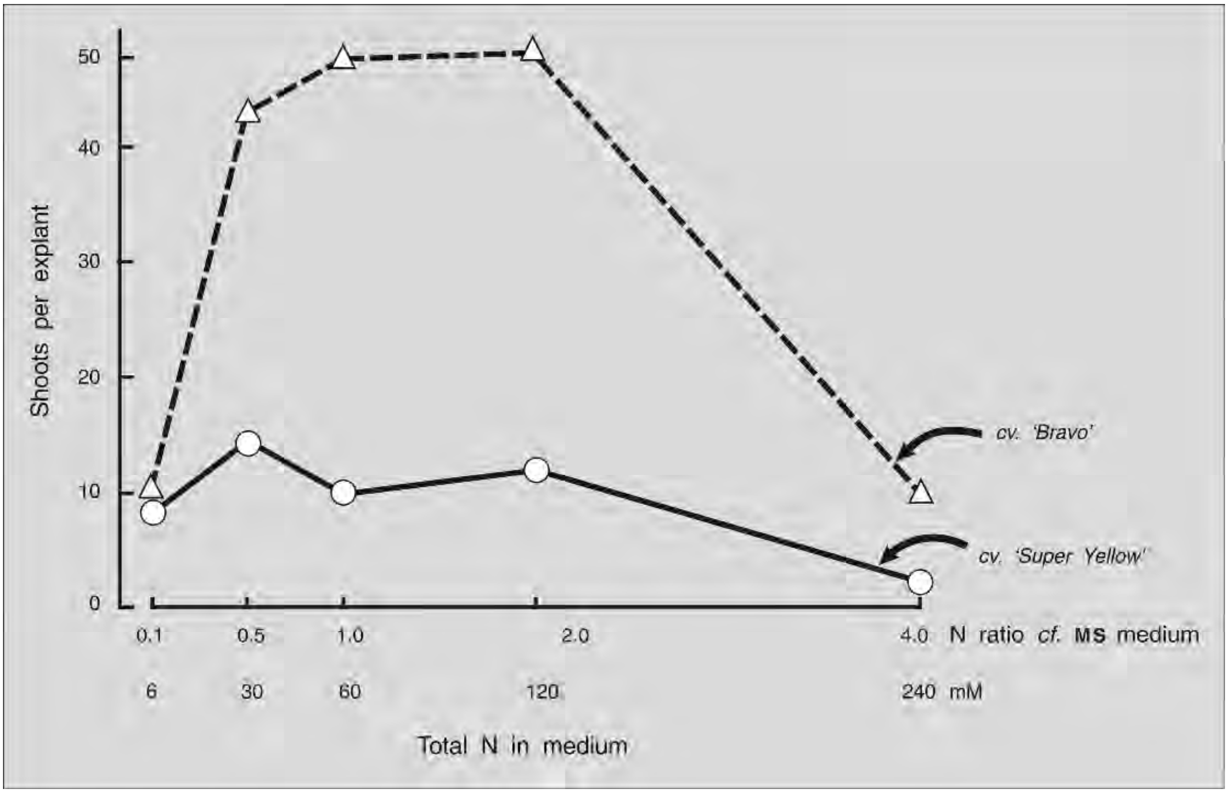

Fig. 3.3 The number of adventitious shoots formed directly on Chrysanthemum explants with increasing total nitrogen concentration in an otherwise normal **MS** medium [from data of Roest and Bokelmann, 1975]

Embryogenesis and embryo growth. It is accepted that the presence of some reduced nitrogen is necessary for somatic embryogenesis in cell and callus cultures (Halperin and Wetherell, 1965; Reinert *et al.,* 1967); but although reduced nitrogen compounds are beneficial to somatic embryo induction, apparently they are not essential until the stage of embryo development (Kamada and Harada, 1979). A relatively high level of both nitrate and ammonium ions then seems to be required. Some workers have also noted enhanced embryogenesis and/or improved embryo growth when media have been supplemented with amino acids in addition to NO_3^- and NH_4^+. Street (1979) thought that an optimum level of NH_4^+ for embryogenesis was about 10 mM (from NH_4Cl) in the presence of 12-40 mM NO_3 (from KNO_3): that is:

[NO_3^- to NH_4^+ ratio, from 55:45 to 80:20; Total N 22-50 mM]

Walker and Sato (1981) obtained no embryogenesis in alfalfa callus in the absence of either ammonium or nitrate ions. Miller's medium [Miller (1961; 1963)] (12.5 mM NH_4^+) supported a high rate of embryogenesis:

[NO_3^- to NH_4^+ ratio, 68:32; Total N 39.1 mM], but only a small number of embryos were produced on Schenk and Hildebrandt (1972) medium (SH):

[NO_3^- to NH_4^+ ratio, 90:10, Total N 27.32 mM], unless it was supplemented with NH_4^+ from either ammonium carbamate, ammonium chloride or ammonium sulphate. An optimal level of ammonium in SH medium was 12.5 mM:

[NO_3^- to NH_4^+ ratio, then 66:34, total N, 37.22], although embryogenesis was still at a high level with 100 mM ammonium ion:

[NO_3^- to NH_4^+ ratio, then, 20:80; total N, 124.72 mM].

By contrast, *Coffea arabica* leaf callus (Sondahl and Sharp, 1977), which was formed on a medium containing MS salts, was induced to become embryogenic by first being cultured on a medium with MS salts (and high auxin):

[NO_3^- to NH_4^+ ratio, 66:34; total N, 30.0 mM],

and then moved to another medium containing MS salts with an extra 2850 mg/l KNO_3:

[NO_3^- to NH_4^+ ratio, 82:18; total N, 58.2 mM] (and low auxin)

Zygotic embryos. The presence of some reduced nitrogen in the growth medium is also required for the continued growth of zygotic embryos in culture. Nitrate alone is insufficient (Mauney *et al.,* 1967; Norstog, 1967, 1973). An optimum concentration of NH_4^+ for the development of barley embryos in culture was 6.4 mM (Umbeck and Norstog, 1979). A similar provision seems to be necessary in most plants for the *in vitro* growth of somatic embryos.

Flower bud formation and growth. Nitrate was essential for the formation of adventitious buds on leaf segments of *Begonia franconis.* The greatest proportion of flower buds was obtained with 5 mM NO_3^- and 1.5 mM NH_4^+. Above this level, NH_4^+ promoted vegetative sprouts (Berghoef and Bruinsma, 1979b). The best *in vitro* growth of *Begonia franconis* flower buds detached from young inflorescences, occurred on a medium with 10-15 mM total nitrogen (NO_3^- to NH_4^+ ratio, 50:50 to 67:33) (Berghoef and Bruinsma, 1979a). Detached flower buds of *Cleome iberidella* were found to grow best *in vitro* with 25 mM total nitrogen (NO_3^- to NH_4^+ ratio, 80:20) (De Jong and Bruinsma, 1974; De Jong *et al.,* 1974), but the complete omission of NH_4NO_3 from MS medium, where the salts had been diluted to their original concentration, promoted the development (but not the initiation) of adventitious floral buds of *Torenia fournieri* (Tanimoto and Harada, 1979, 1981, 1982).

Effect on the action of growth regulants. The ratio of NO_3^- to NH_4^+ present in the culture medium has been found to affect the activity of plant growth substances and plant growth regulators. The mechanisms by which this occurs are not fully elucidated. It has been noted, for example, that cells will divide with less added cytokinin when the proportion of reduced nitrogen is reasonably high. To induce tobacco protoplasts to divide, it was necessary to add 0.5-2 mg/l BAP to a medium containing only NO_3^- nitrogen. The presence of glutamine or NH_4^+ in the medium together with NO_3^-, reduced the cytokinin requirement, and division proceeded without any added cytokinin when urea, NH_4^+, or glutamine were the sole N-sources of the medium (Meyer and Abel, 1975b). Sargent and King (1974) found that soybean cells were dependent on cytokinin when cultured in a medium containing NO_3^- nitrogen, but independent of cytokinin when NH_4^+ was present as well.

The relative proportion of nitrate and ammonium ions also affects the response of cells to auxin growth regulators in terms of both cell division and morphogenesis. It is possible that this is through the control of intracellular pH (see below). Carrot cultures that produce somatic embryos when transferred from a high- to a low-auxin medium, can also be induced into embryogenesis in a high-auxin medium, if it contains adequate reduced nitrogen. Only root initials are formed in high-auxin media which do not contain reduced nitrogen (Halperin, 1967). The number of plants regenerated from rice callus grown on Chu *et al.,* (1975) medium containing 0.5 mg/l 2,4-D, depended on the ratio of NO_3^- to NH_4^+. It was high in the unaltered medium (ratio 4:1), but considerably less if, with the same total N, the ratio of the two ions was changed to 1:1 (Grimes and Hodges, 1990).

Cells of *Antirrhinum majus* regenerated from isolated protoplasts were stimulated to divide with a reduced quantity of auxin in a medium containing 39.77 mM total nitrogen

[NO_3^- to NH_4^+ ratio, 39:77 (2.98)]

by adding further ammonium ion to give a total nitrogen content of 54.72 mM

[NO_3^- to NH_4^+ ratio, 54:46 (1.19)].

or, alternatively, 400 mg/l of casein hydrolysate (Poirier-Hamon *et al.,* 1974).

In experiments of Koetje *et al.,* (1989) and Grimes and Hodges (1990) (Table 3.5), when the NO_3^- to NH_4^+ ratio in N6 medium was 80:20, there was a strong dose response curve to the auxin 2,4-D with 0.5 mg/l being the best concentration to induce embryogenesis in *Oryza sativa* callus; if the medium was modified, so that the NO_3^- to NH_4^+ ratio was 66:34 or 50:50, 2,4-D was less effective, and there was little difference in the number of plants regenerated between 0.5 and 3 mg/l 2,4-D. The ratio of NO_3^- to NH_4^+ therefore seemed either to to alter the sensitivity of cells to the auxin, or to affect its uptake or rate of metabolism.

Walker and Sato (1981) also found that the proportion of ammonium ion in the medium can influence the way in which growth regulants control morphogenesis. Having been placed for 3 days on a medium which would normally induce root formation [Schenk and Hildebrandt (1972) medium containing 5 mM 2,4-D and 50 mM kinetin], suspension cultured cells were subsequently plated on a modification of the same medium (which contains 24.8 mM NO_3^-) without regulants, in which the concentration of NH_4^+ had been adjusted to various levels. Table 3.6 shows that the morphogenesis experienced, depended on the concentration of ammonia in the regeneration medium. Media containing high levels of ammonium ion would have tended to become acid, especially as the extra ammonium was added as ammonium sulphate. Possibly this affected the uptake or action of the regulants?

2.1.10. Addition of amino acids

Amino acids can be added to plant media to satisfy the requirement of cultures for reduced nitrogen, but as they are expensive to purchase, they will only be used in media for mass propagation where this results in improved results. For most tissue culture purposes, the addition of amino acids may be unnecessary, providing media contain adequate amounts, and correct proportions, of nitrate and ammonium ions. For example, Murashige and Skoog (1962) found that when cultures were grown on media such as Heller (1953; 1955), Nitsch and Nitsch (1956) N1, and Hildebrandt *et al.,* (1946) Tobacco, containing sub-optimal amounts of inorganic chemicals, a casein hydrolysate (consisting mainly of a mixture of amino acids, see later) substantially increased the yield of tobacco callus, whereas it gave only marginal increases in yield when added to their revised MS medium. Arginine (0.287 mM) increased the growth of sugar cane callus and suspension cultures grown on Nickell and Maretzski (1969) medium (Nickell and Maretzski, 1969) but was without effect on cultures of this plant grown on a medium based on Scowcroft and Adamson (1976) CS5 macronutrients (Larkin, 1982).

It is noticeable from the literature that organic supplements (particularly amino acids) have been especially beneficial for growth or morphogenesis when cells or tissues were cultured on media such as White (1943a), which do not contain ammonium ions. White (1937) and Bonner and Addicott (1937), for example, used known amino acids to replace the variable mixture provided by yeast extract. For the culture of *Picea glauca* callus, Reinert and White (1956) supplemented Risser and White (1964) medium with 17 supplementary amino acids, and similar, or greater numbers, were used by Torrey and Reinert (1961) and Filner (1965) in White (1943) medium for the culture of carrot, *Convolvulus arvensis*, *Haplopappus gracilis* and tobacco tissues.

Dependence on the nitrate to ammonium ratio. Grimes and Hodges (1990) found that when both NO_3^- and NH_4^+ are present in the medium, the response to organic nitrogen depends on the ratio of these two ions. Twice as many plants were regenerated from embryogenic rice callus when 1g/l CH was added to Chu *et al.,* (1975) N6 medium, providing the proportion of NO_3^- to NH_4^+ was also changed to 1 (50:50). There was little response to CH with the same amount of total N in the medium, if the NO_3^-/NH_4^+ ratio was 4 (i.e. 80%:20%, as in the original medium), or more.

2.1.11. Amino acids as the sole N source

As most of the inorganic nitrogen supplied in culture media is converted by plant tissues to amino acids, which are then assimilated into proteins, it should be possible to culture plants on media in which amino acids are the only nitrogen source. This has been demonstrated: for example, *Nicotiana tabacum* callus can be cultured on MS salts lacking NO_3^- and NH_4^+ (but with an extra 20.6 mM K^+), if 0.1 mM glycine, 1mM arginine, 2 mM aspartic acid and 6 mM glutamine are added (Muller and Grafe, 1978); wild carrot suspensions can be grown on a medium

Table 3.6 The effect of total nitrogen, and the ratio of NO_3^- to NH_4^+ on the type of organ produced by alfalfa cells which have been subcultured on a root-inducing medium (Walker and Sato, 1981)

Type of organ produced	NH_4^+ (mM)	NO_3^- (mM)	Ratio of NO_3^- to NH_4^+
Roots	<2.5	27.2	100:0 to 91:9
Roots *and* somatic embryos	12.5-37.5	37.3-62.3	66:34 to 40:60
Somatic embryos	50-100	74.7-124.7	33:67 to 20:80

containing glutamine or casein hydrolysate as the sole nitrogen source (Anderson, 1976). Amino acids provide plant cells with an immediately available source of nitrogen, and uptake can be much more rapid than that of inorganic nitrogen in the same medium (Thom *et al.,* 1981). Only the L- form of amino acids is biologically active.

Amino acids can also provide reduced nitrogen in culture media in place of NH_4^+ and as a supplement to NO_3^-. However they are usually employed as minor additions to media containing both NH_4^+ and NO_3^-. Uptake of amino acids into cultured tissues causes a decrease in the pH of the medium, similar to that which occurs when NH_4^+ ions are absorbed.

Sugar-based amines such as glucosamine and galactosamine can also serve as a source of reduced nitrogen in morphogenesis (Margara, 1969a; Margara and Leydecker, 1978).

2.1.12. Biologically-active amino acids

Amino acids are classified according to their stereoisomers and according to the relative positions of the amino group and the acidic radical. Only the L- isomers of the α-amino acids are important for plant tissue culture media. They have the general structure:

$$\begin{array}{c} \quad NH_2 \\ \quad | \\ R\text{-}CH\text{-}\ COOH \end{array}$$

β-Amino acids are present in plants but tend to result from secondary metabolism. They have the general structure:

$$\begin{array}{c} \quad NH_2 \\ \quad | \\ R\text{-}CHCH_2\text{-}\ COOH \end{array}$$

(where R = functional groups)

Unfortunately the particular amino acid, or mixture of amino acids, which promotes growth or morphogenesis in one species, may not do so in another. For instance, L-α-alanine, glutamine, asparagine, aspartic acid, glutamic acid, arginine and proline could serve as a source of reduced nitrogen in a medium containing 20 mM NO_3^-, and were effective in promoting embryogenesis in *Daucus carota* callus and suspensions, but lysine, valine, histidine, leucine and methionine were ineffective (Kamada and Harada, 1982).

Competitive inhibition. Some amino acids are growth inhibitory at fairly low concentrations and this is particularly observed when mixtures of two or more amino acids are added to media. Inhibition is thought to be due to the competitive interaction of one compound with another. In oat embryo cultures, phenylalanine and L-tyrosine antagonise each other, as do L-leucine and DL-valine; DL-isoleucine and DL-valine, and L-arginine and L-lysine (Harris, 1956). Lysine and threonine often exert a co-operative inhibition when present together, but do not inhibit growth when added to a medium singly (Cattoir-Reynaerts *et al.,* 1981).

Glycine. Glycine is an ingredient of many media. It has usually been added in small amounts, and has been included by some workers amongst the vitamin ingredients. Despite frequent use, it is difficult to find hard evidence that glycine is really essential for so many tissue cultures, but possibly it helps to protect cell membranes from osmotic and temperature stress (Orczyk and Malepszy, 1985).

White (1939) showed that isolated tomato roots grew better when his medium was supplemented with glycine rather than yeast extract and that glycine could replace the mixture of nine amino acids that had been used earlier. It was employed as an organic component by Skoog (1944) and continued to be used in his laboratory until the experiments of Murashige and Skoog (1962). They adopted the kinds and amounts of organic growth factors specified by White (1943a) and so retained 2 mg/l glycine in their medium without further testing.

Linsmaier and Skoog (1965), furthering the study of medium components to organic ingredients, omitted glycine from MS medium and discovered that low concentrations of it had no visible effect on the growth of tobacco callus, while at 20 mg/l it depressed growth. No doubt the success of MS medium has caused the 2 mg/l glycine of Skoog (1944) to be copied in many subsequent experiments. Many workers overlook the later Linsmaier and Skoog paper.

Casein and other protein hydrolysates. Proteins, which have been hydrolysed by acid, or enzymes, and so broken down into smaller molecules, are less costly than identified amino acids. The degree of degradation varies: some protein hydrolysates consist of mixtures of amino acids together with other nitrogenous compounds such as peptide fragments, vitamins, and elements which might (if they can form inorganic ions, or are associated with organic compounds that can be taken

up by plant tissues) be able to serve as macro- or micro-elements. Peptones are prepared from one or several proteins in a similar fashion but generally consist of low molecular weight proteins. Although protein hydrolysates are a convenient source of substances which may promote plant growth, they are by nature relatively undefined supplements. The proportion of individual amino acids in different hydrolysates depends on the nature of the source protein and the method by which the product has been prepared.

The hydrolysate most often used in culture media is that of the milk protein, casein, although lactalbumin hydrolysate has been employed (La Motte and Lersten, 1971). Peptones and tryptone have been used less frequently, but there are reports of their having been added to media with advantage (e.g. Muralidhar and Mehta, 1982; Pierik *et al.,* 1988). Casein hydrolysates can be a source of calcium, phosphate, several microelements, vitamins and, most importantly, a mixture of up to 18 amino acids. Several casein hydrolysates (CH) are available commercially but their value for plant tissue culture can vary considerably. Acid hydrolysis can denature some amino acids and so products prepared by enzymatic hydolysis are to be preferred. The best can be excellent sources of reduced nitrogen, as they can contain a relatively large amount of glutamine.

Casein hydrolysate produces an improvement in the growth of *Cardamine pratensis* and *Silene alba* suspensions, only if the medium is deficient in phosphorus. Glutamine has the same effect; it is the most common amino acid in CH, and its synthesis requires ATP. For these reasons, Bister-Miel *et al.,* (1985) concluded that CH overcomes the shortage of glutamine when there is insufficient phosphorus for adequate biosynthesis.

However several investigators have concluded that casein hydrolysate itself is more effective for plant culture than the addition of the major amino acids which it provides. This has led to speculation that CH might contain some unknown growth-promoting factor (Inoue and Maeda, 1982). In prepared mixtures of amino acids resembling those in CH, competitive inhibition between some of the constituents is often observed. For instance, the induction of embryogenesis in carrot cell suspensions on a medium containing glutamine as the only nitrogen source, was partly inhibited by the further addition of L-amino acids similar in composition to those in CH. This suppression was mainly caused by the L-tyrosine in the mixture (Anderson, 1976).

There may be a limit to the amount of CH, which can be safely added to a medium. Anstis and Northcote (1973) reported that the brand of CH known as 'N-Z-amine', can produce toxic substances if concentrated solutions are heated, or if solutions are frozen and thawed several times. Possibly these are reasons why mixtures of amino acids occasionally provide more valuable supplements than CH. *Nicotiana tabacum* callus grew better on a nitrogen-free MS medium when a mixture of the amino acids L-glutamine (6 mM), L-aspartic acid (2 mM), L-arginine (1 mM) and glycine (0.1 mM) was added, rather than 2 g/l casein hydrolysate (which would have provided about 2 mM glutamine, 0.6 mM aspartic acid, 0.2 mM arginine and 0.3 mM glycine) (Muller and Grafe, 1978).

2.1.13. Beneficial effects of amino acid additions

Improved growth. The growth rate of cell suspensions is frequently increased by the addition of casein hydrolysate or one or more amino acids (particularly glutamine) to media containing both nitrate and ammonium ions. Some workers have included a mixture of several amino acids in their medium without commenting on how they improved growth. In other cases the benefit resulting from a specific compound has been clearly shown. The lag phase of growth in suspensions of *Pseudotsuga menziesii* cultured on Cheng (1977; 1978) medium was eliminated by the addition of 50 mM glutamine, and the final dry weight of cells was 4 times that produced on the unammended medium (Kirby, 1982). Similarly the rate of growth of *Actinidia chinensis* suspensions was improved by the addition of 5mM glutamine (Suezawa *et al.,* 1988) and those of *Prunus amygdalus* cv. 'Ferragnes' could not be maintained unless 0.2% casamino acids was added to the medium (Rugini and Verma, 1982). Molnar (1988b) found that the growth of *Brassica nigra* cell suspensions was improved by adding 1-4 g/l CH or a mixture of 4 mM alanine, 4 mM glutamine and 1 mM glutamic acid. In this case the medium contained MS salts (but less iron) and B5 vitamins.

Amino acid supplements have also been used to boost the rate of growth of callus cultures. For instance, Short and Torrey (1972) added 5 amino acids and urea to a medium containing MS salts for the culture of pea root callus, and Sandstedt and Skoog (1960) found that aspartic and glutamic acids promoted the growth of tobacco callus as much as a mixture of several amino acids (such as found in yeast extract). Glutamic acid seemed to be primarily responsible for the growth promotion of sweet clover

callus caused by casein hydrolysate on a medium containing 26.6 mM NO_3^-, 12.5 mM NH_4^+ and 2.0 mM PO_4^{3-} (Taira *et al.,* 1977).

Amino acids are often added to media for protoplast culture. It was essential to add 2 mM glutamine and 2 mM asparagine to a medium containing MS salts, to obtain cell division, colony growth and plantlet differentiation from *Trigonella* protoplasts (Shekhawat and Galston, 1983).

Shoot cultures. Many shoot cultures are grown on MS medium containing glycine, although in most cases the amino acid is probably not an essential ingredient. Usually it is unnecessary to add amino acids to media supporting shoot cultures, but methionine may represent a special case. Druart (1988) found that adding 50-100 mg/l L-methionine to the medium seemed to stimulate cytokinin activity and caused cultures of *Prunus glandulosa* var. *sinensis* to have high propagation rates through several subcultures. This promotive effect of L-methionine was thought to be due to it acting as a precursor of ethylene (see Chapter 7). Glutamine inhibited the growth of apical domes excised from *Coleus blumei* shoots (Smith, 1981) and 50-100 mg/l glutamic acid inhibited shoot growth, the formation of axillary buds and shoot proliferation in cultures of woody plants (Druart, loc. cit.).

L-Citrulline is an important intermediate in nitrogen metabolism in the genus *Alnus*. The addition of 1.66 mM (4.99 mM NH_2) to WPM medium improved the growth of *A. cordata* and *A. subcordata* shoot cultures (Cremiere *et al.,* 1987).

Contaminants grow more rapidly on media containing amino acids. Casein hydrolysate is therefore sometimes added to the media for Stage I shoot cultures so that infected explants can be rejected quickly (Schulze, 1988). The health of shoots grown from seedling shoot tips of *Feijoa* (*Acca) sellowiana* was improved when 500 mg/l CH was added to Boxus (1974) medium (which does not contain ammonium ions) (Bhojwani et al., 1987).

Organogenesis. The presence of amino acids can enhance morphogenesis, either when they provide the only source of reduced nitrogen, or when they are used as a supplement to a medium containing both NO_3^- and NH_4^+. In a medium containing 25 mM nitrate, but no NH_4^+, direct adventitious shoot formation on cauliflower peduncle explants was induced by the addition of a mixture of the amino acids asparagine, proline, tyrosine and phenylalanine, each at a concentration of only 0.1 mM. (Margara, 1969b). A high rate of adventitious shoot regeneration and embryogenesis, from *Beta vulgaris* petioles or petiole callus, was achieved on a medium comprised of several amino acids and a complex vitamin mixture with MS salts (Freytag *et al.,* 1988). The addition of CH to MS medium was found to be essential for shoot formation from callus (Chand and Roy, 1981).

Adding only 1-10 mg/l of either L-leucine or L-isoleucine to Gamborg *et al.,* (1968) B5 medium, decreased callus growth of *Brassica oleracea* var *capitata*, but increased adventitious shoot formation. Basu *et al.,* (1989) thought that this might be due to these amino acids being negative effectors of threonine deaminase (TD) enzyme, the activity of which was diminished in their presence. Threonine, methionine and pyruvic acid, which increased callus growth in this species, enhanced TD activity.

There are several examples of the amino acid L-asparagine being able to stimulate morphogenesis. This may be because it too can be a precursor of ethylene (Durzan, 1982), the biosynthesis of which may be increased by greater substrate availability. Kamada and Harada (1977) found that the addition of 5 mM L-asparagine stimulated both callus and bud formation in stem segments of *Torenia fournieri*, while alanine (and, to a lesser extent, glutamic acid) increased flower bud formation from *Torenia* internode segments when both an auxin and a cytokinin were present. An increase in the number of adventitious buds formed on the cotyledons and hypocotyl of *Chamaecyparis obtusa* seedlings occurred when 1.37 mM glutamine and 1.51 mM asparagine were added together to Campbell and Durzan (1975) medium, but not when they were supplied on their own (Ishii, 1986). L-asparagine was also added to MS medium by Green and Phillips (1975), to obtain plant regeneration from tissue cultures of maize; adding it to Finer and Nagasawa (1988) 10A4ON medium caused there to be more embryogenic clumps in *Glycine max* suspension cultures (Finer and Nagasawa, 1988).

Amino acid additions do not invariably enhance morphogenesis. Supplementing Linsmaier and Skoog (1965) medium with 0.5-5 mM glutamine, caused callus of *Zamia latifolia* to show greatly decreased organogenesis (Webb and Rivera, 1981) and ammending Linsmaier and Skoog (1965) medium with 100 mg/l CH, prevented adventitious shoot formation from stem internode callus of apple and cherry rootstocks (James *et al.,* 1984).

Embryogenesis. The presence the ammonium ion is usually sufficient for the induction of

embryogenesis in callus or suspension cultures containing NO_3^-, but on media where NH_4^+ is lacking [e.g. White (1954)], casein hydrolysate, or an amino acid such as alanine, or glutamine, is often promotory (Ranga Swamy, 1958; Ammirato and Steward, 1971; Street, 1979). For embryogenesis in carrot cultures, Wetherell and Dougall (1976) have shown that in a medium containing potassium nitrate, reduced nitrogen in the form of ammonium chloride matched the effectiveness of an equivalent concentration of nitrogen from casein hydrolysate. Casein hydrolysate could be replaced by glutamine, glutamic acid, urea or alanine. Suspensions of wild carrot cells grew and produced somatic embryos on a medium containing either glutamine or CH as the sole nitrogen source (Anderson, 1976).

There have also been many reports of embryogenesis being promoted by the addition of casein hydrolysate, or one or more specific amino acids, when both NO_3^- and NH_4^+ were available in the medium. Some examples are given in Table 3.7. In many cases, embryogenic callus and/or embryo formation did not occur without the presence of the amino acid source, suggesting that without amino acid, the medium was deficient in NH_4^+ or total nitrogen. Armstrong and Green (1985) found that the frequency of friable callus and somatic embryo formation from immature embryos of *Zea mays* increased almost linearly with the addition of up to 25 mM proline to Chu *et al.,* (1975) N6 medium

[total N, 34.99 mM; NO_3/NH_4 ratio, 3.99],

but there was no benefit from adding proline to MS medium (containing 150 mg/l asparagine hydrate).

[total N, 60.01 mM; NO_3/NH_4 ratio, 1.91]

The growth of somatic embryos can also be affected by the availability of reduced nitrogen. That of *Coronilla varia* embryos was poor on Gamborg *et al.,* (1968) B5 medium (Total N 26.74 mM; NH_4^+ 2.02 mM), unless 10 mM asparagine or 20 mM NH_4Cl was added to the medium, or unless the embryos were moved to Saunders and Bingham (1972) BOi2Y medium, which has 37.81 mM total inorganic N, 12.49 mM NH_4^+ and 2000 mg/l casein hydrolysate (approx. 9.9 mM NH_4^+ equivalence) (Moyer and Gustine, 1984). However, the germination of *Triticum aestivum* somatic embryos was completely prevented by adding 800 mg/l CH to MS medium (Ozias Akins and Vasil, 1982; Carman *et al.,* 1988).

Culture of immature cotyledons. Young storage cotyledons isolated from immature zygotic embryos accumulate protein efficiently when cultured with amino acids in a medium without nitrate and ammonium ions. A medium such as that of Millerd *et al.* (1975), or of Thompson *et al.,* (1977) is normally used, but where the effect of different amino acids on protein assimilation is being studied, the amino acid content of the medium is varied. Glutamine is often found to be the most efficient nitrogen source for this purpose (Thompson *et al.,* 1977; Haga and Sodek, 1987), but protein increase from culture with asparagine and glutamate (glutamic acid) is usually also significant (Lea *et al.,* 1979).

2.1.14. Causes of the stimulatory effect of amino acids

We may conclude therefore, that for many cultural purposes, amino acids are not essential media components; but their addition as identified pure compounds, or more cheaply through casein hydrolysates, can be an easy way of ensuring against medium deficiency, or of providing a source of nitrogen that is immediately available to cultured cells or tissues. An observation by Murashige and Skoog (1962) that the presence of casein hydrolysate allowed vigorous organ development over a broader range of IAA and kinetin levels, may be of significance.

In gram moles per litre, amino acids can be a much more efficient source of reduced nitrogen than ammonium compounds. For instance, the mixture of amino acids provided by 400 mg/l of casein hydrolysate (containing at most as much reduced nitrogen as 3.3 mM NH_4^+) was as effective as 14.95 mM NH_4Cl in stimulating the division of protoplast-derived cells of *Antirrhinum* (Poirier-Hamon *et al.,* 1974).

Why should this be, and why can additions of amino acids (sometimes in comparatively small amounts) stimulate growth or morphogenesis when added to media, which already contain large amounts of NH_4^+? Some hypotheses, which have been advanced are:

- **Conservation of ATP - alleviating phosphate deficiency.** Durzan (1982) pointed out that when plant tissues take up the ammonium ion, they consume adenosine tri-phosphate (ATP) in converting it to amino acids. If suitable amino acids are available from the medium, some of ATP may be conserved. Bister-Miel *et al.,* (1985) noted that CH promoted growth in cultures where phosphate became growth-limiting. They suggested that amino acids compensated for phosphate deficiency. With the plant well supplied with amino acids, some of the phosphate, which is normally used for ATP production can be diverted to other uses. Several

Table 3.7 Some examples of the promotion of embryogenesis by amino acids in media containing NO_3^- and NH_4^+

	Type of culture	Basal medium used	Amino acid supplements	Reference
Aesculus hippocastrum	Zygotic embryo callus	MS	CH (250 mg/l) + Proline (250 mg/l)	Radojevic (1988)
Dactylis glomerata	Suspension-derived callus	Schenk and Hildebrandt (1972)	CH (1.5 g/l)	Gray *et al.* (1984)
Daucus carota	Hypocotyl callus	Gamborg *et al.* (1968) B5	Proline (100 mM) + Serine (100 mM)	Nuti Ronchi *et al.* (1984)
Dioscorea rotundata	Zygotic embryo callus	MS	CH (1 g/l)	Osifo (1988)
Glycine max	Suspension	Kartha *et al.* (1974a)	L-asparagine (5 mM)	Finer and Nagasawa (1988)
Gossypium klotzschianum	Suspension	Gamborg *et al.* (1968) B5	Glutamine (10 mM)	Price and Smith (1979a,b)
Larix decidua	Gametophyte callus	Litvay *et al.* (1981) LM	CH (1 g/l) + Glutamine (500 mg/l)	Nagmani and Bonga (1985)
Nigella sativa	Roots or leaf callus	MS	CH (100 – 500 mg/l)	Bannerjee and Gupta (1976)
Trigonella foenum-graecum	Leaf callus	MS	CH (50 mg/l) (500 mg/l was inhibitory)	Gupta *et al.* (1987)
Triticum aestivum	Anther	Chu and Hill (1988) MN6	Serine, proline, arinine, aspartic acid and alanine (each at 40 mg/l) + glutamine (400 mg/l)	Chu and Hill (1988)
Vitis vinifera	Anther	½ MS	CH (250 mg/l)	Mauro *et al.* (1986);
Zea mays	Zygotic embryo callus	Chu *et al.* (1975) N6	Proline (20-25 mM)	Kamo *et al.* (1985) Armstrong and Green (1985)

authors have pointed out that CH itself is also a source of phosphate. For example, Bridson (1978) and some chemical catalogues, show that some casein hydrolysates normally contain about 1.3g P_2O_5 per 100g. The addition of 2 g/l of CH will therefore increase the phosphate content of MS medium by 11% and that of White (1954) medium by 44% (assuming complete phosphate availability).

- **A capacity to act as chelating agents.** Some amino acids can act as chelating agents (see later in section on chelates)
- **Enhanced nitrogen assimilation.** Glutamine and glutamic acid are directly involved in the assimilation of NH_4^+. A direct supply of these amino acids should therefore enhance the utilization of both nitrate and ammonium nitrogen and its conversion into amino acids.
- **A replacement for toxic ammonium ions.** Certain plant tissues are particularly sensitive to NH_4^+. Ochatt and Caso (1986) and Ochatt and Power (1988a, b) found that protoplasts of *Pyrus* spp. would not tolerate the ion, and that to obtain sustained cell division it was necessary to eliminate it from MS medium, and use 50 mg/l casein hydrolysate as a source of reduced nitrogen. CH can however be extremely toxic to freshly isolated protoplasts of some species and varieties of plants (Ranch and Widholm, 1980; Russell and McCown, 1988). Conifer tissues too are unable to cope with high

concentrations of NH_4^+, but cultures can be supplied with equivalent levels of reduced nitrogen in the form of amino acids without the occurrence of toxicity (Durzan, 1982). In soybean suspension cultures, the high level of ammonium in MS medium has been shown to inhibit isocitrate dehydrogenase (a Krebs' cycle enzyme) and glutamine synthetase, which contribute to the conversion of NH_4^+ to glutamine (Gamborg and Shyluk, 1970).

- **Adjustment of intracellular pH.** As intracellular pH is important for the activation of sea urchin eggs, and amino acids can promote embryogenesis, Nuti Ronchi *et al.,* (1984) speculated that the uptake and assimilation of amino acids might help to regulate cellular pH in plants.

As mentioned before, there is commonly a minimum inoculation density below which growth cannot be initiated *in vitro*. This minimum varies according to both the source of the cells and the nature of the medium. It can usually be lowered by employing a 'conditioned' medium (i.e. a fresh medium into which the products of another medium in which cells are actively growing, have been added). Alternatively, initial growth at low densities can be supported by the close presence of other actively growing plant cells ('nurse cultures'). Compounds responsible for this effect must be freely diffusible from living cells and could include growth substances, reducing sugars, vitamins and amino acids. Addition of such supplements has been found to overcome the inhibited growth of some cells at low densities (Kao and Michayluk, 1975).

2.2. PHOSPHATE

Phosphorus is a vital element in plant biochemistry. It occurs in numerous macromolecules such as nucleic acids, phospholipids and co-enzymes. It functions in energy transfer via the pyrophophate bond in ATP. Phosphate groups attached to different sugars provide energy in respiration and photosynthesis and phosphate bound to proteins regulates their activity. Phosphorus is absorbed into plants in the form of the primary or secondary orthophosphate anions $H_2PO_4^-$ and HPO_4^{2-} by an active process, which requires the expenditure of respiratory energy. Phosphate, in contrast to nitrate and sulphate, is not reduced in plants, but remains in the highly oxidized form. It is used in plants as the fully oxidized orthophosphate (PO_4^{3-}) form.

In culture media the element is provided as soluble potassium mono- and di-hydrogen phosphates. The di- and mono-valent phosphate anions respectively provided by these chemicals are interconvertible in solution depending on pH. Monovalent $H_2PO_4^-$ predominates at pH values below 7, characteristic of most tissue culture media, and it is this ion, which is most readily absorbed into plants (Devlin, 1975). Conversion of $H_2PO_4^-$ into divalent HPO_4^{2-} begins to occur as solutions become more alkaline. The divalent ion is said to be only sparingly available to plants but Hagen and Hopkins (1955) and Jacobsen *et al.,* (1958) thought that its absorption could be significant, because even though the ion is normally at a relatively low concentration in nutrient solutions, its affinity with the site of absorption is greater than that of the mono-valent form. Trivalent PO_4^{3-}, which appears in alkaline solutions, is not generally absorbed by plants.

In some early tissue culture media, all (e.g. Bouharmont, 1961), or part (e.g. Vacin and Went, 1949) of the phosphorus was supplied as sparingly-soluble phosphates. A slow rate of phosphorus availability seems to be possible from such compounds. The optimum rate of uptake of phosphate (HPO_4^{2-}) into cultured *Petunia* cells occurred at pH 4 (Chin and Miller, 1982) but Zink and Veliky (1979) did not observe any decline in the absorption of phosphate by *Ipomoea* suspension cultures at pH 6.5, when HPO_4^{2-} and $H_2PO_4^-$ were present in approximately equal concentrations. Plant tissue cultures secrete phosphatase enzymes into the medium (Ciarrocchi *et al.,* 1981), which could release phosphate ions from organic phosphates.

In the cytoplasm, phosphate is maintained at a constant concentration of 5-10 mM, more or less independent of the external concentration. Phosphate in the vacuole fluctuates according to the external concentration but does not increase above 25 mM (Schachtman *et al.,* 1998). When there is a high supply of phosphate and it is taken up at rates that exceed the demand, a number of processes act to prevent toxic phosphate concentrations, among others storage into inorganic compounds such as phytic acid. High concentrations of dissolved phosphate can depress growth, possibly because calcium and some microelements are precipitated from solution and/or their uptake reduced. In *Arabidopsis thaliana*, four different phophate transporter genes have been isolated (*APT1-4*). *In vivo*, the genes are predominantly expressed in the roots and their expression is constitutive or induced by phosphate starvation. Overexpression of *APT1* gene in tobacco cell cultures increased the rate of phosphate uptake (Mitsukawa *et al.,* 1997).

Although the concentration of phosphate introduced into plant culture media has been as high as 19.8 mM, the average level is 1.7 mM and most media contain about 1.3 mM. However many reports indicate that such typical levels may be too low for some purposes. When phosphate is depleted from MS medium, there is an increase in free amino acids in *Catharanthus roseus* cells, because protein synthesis has ceased and degradation of proteins is occurring (Ukaji and Ashihara, 1987). Phosphate (starting concentration 2.64 mM) and sucrose were the only nutrients completely depleted in *Catharanthus roseus* batch suspension cultures, and the period of growth could be prolonged by increasing the levels of both (MacCarthy *et al.,* 1980). MS medium contains only 1.25 mM phosphate which may be insufficient for suspension cultures of some plants. The phosphate in MS medium is insufficient for *Cardamine pratensis* suspension cultures, all having been absorbed in 5 days: it is however adequate for *Silene alba* suspensions (Bister-Miel *et al.,* 1985).

The phosphate in MS medium is also inadequate for static cultures of some plants, or where a large amount of tissue or organs are supported on a small amount of medium (for example where many separate shoots are explanted together in a static shoot culture). The concentration of the ion is then likely to be reduced almost to zero over several weeks (Barroso *et al.,* 1985; Singha *et al.,* 1987; Lumsden *et al.,*1990). Insufficient levels of phosphate were present from MS during culture of *Hemerocallis*, *Iris* and *Delphinium* (Leiffert *et al.,* 1995). Although growth can continue for a short while after the medium is depleted of phosphate, for some purposes it has been found to be beneficial to increase the phosphate concentration of MS to 1.86 mM (Jones and Murashige, 1974), 2.48 mM (Murashige *et al.,* 1972; Murashige, 1974; Jakobek *et al.,* 1986), 3.1 mM (Miller and Murashige, 1976) or 3.71 mM (Thorpe and Murashige, 1968, 1970), for example, to induce adventitious shoot formation from callus, or to increase the rate of shoot multiplication in shoot cultures. It should be noted that there is *in vivo* a significant retranslocation of phosphate from older leaves to the growing shoot (Schachtman *et al.,* 1998). Retranslocation also occurs in tissue culture. In *Dahlia* culture in liquid medium, phosphate is almost completely taken up after 2 weeks (Fig. 3.4a). In spite of this, the concentration in tissues formed after the exhaustion is 'normal' (Fig. 3.4b). The depletion of phosphate early during culture has also a major effect on the pH of tissue culture media in which added phosphate is the major buffering component. When phosphate levels are increased to obtain a more rapid rate of growth of a culture, it can be advisable to investigate the simultaneous enhancement of the level of *myo*-inositol in the medium

2.3. POTASSIUM

Potassium is the major cation (positive ion) within plants reaching in the cytoplasm and chloroplasts concentrations of 100 – 200 mM. The biphasic uptake kinetics suggest two uptake systems: a high-affinity and a low-affinity one. K^+ is not metabolised. It contributes significantly to the osmotic potential of cells. K^+ counterbalances the negative charge of inorganic and organic anions. It functions in cell extension through the regulation of turgor, it has a major role in stomatal movements and functions in long-distance nutrient flow. Potassium ions are transported quickly across cell membranes and two of their major roles are regulating the pH and osmotic environment within cells. Potassium, calcium, sodium and chloride ions conserve their electrical charges within the plant, unlike the cation NH_4^+ and the anions NO_3^-, SO_4^{2-}, and $H_2PO_4^-$, which are rapidly incorporated into organic molecules. In intact plants, potassium ions are thought to cycle. They move, associated with cations (particularly NO_3^-), upwards from the roots in the xylem. As nitrate is reduced to ammonia and assimilated, carboxylic acid ions (RCO_3^-, malate) are produced. These become associated with the released K^+ ions and are transported in the phloem to the roots, where they are decarboxylated, releasing K^+ for further anion transport (Ben-Zioni *et al.,* 1971). Carboxylate transported to the roots gives rise to OH^- ion, which is excreted into the soil (or medium) to counterbalance NO_3^- uptake (Touraine *et al.,* 1988). Potassium ions will clearly have a similar role in cultured tissues, but obvious transport mechanisms will usually be absent.

Many proteins show a high specificity for potassium which, acting as a cofactor, alters their configuration so that they become active enzymes. Potassium ions also neutralise organic anions produced in the cytoplasm, and so stabilise the pH and osmotic potential of the cell. In whole plants, deficiency of potassium results in loss of cell turgor, flaccid tissues and an increased susceptibility to drought, salinity, frost damage and fungal attack. A high potassium to calcium ratio is said to be

characteristic of the juvenile stage in woody plants (Boulay, 1987). Potassium deficiency in plant culture media is said to lead to hyperhydricity (Pasqualetto *et al.,* 1988), and a decrease in the rate of absorption of phosphate (Chin and Miller, 1982). However quite wide variations in the potassium content of MS medium had little effect on the growth or proliferation of cultured peach shoots (Loreti *et al.,* 1988).

Lavee and Hoffman (1971) reported that the optimum rate of callus growth of two apple clones was achieved in a medium containing 3.5 mM K^+: when the concentration was much higher than this, or when it was less than 1.4 mM, the callus grew less vigorously. However, the growth rate of wild carrot suspensions was said by Brown *et al.,* (1976) to be at, or near, the maximum when K^+ concentration was 1 mM: for embryogenesis 10-50 mM K^+ was required. Uptake of potassium into plants is reduced in the absence of calcium (Devlin, 1975).

Within a large sample of different macronutrient compositions, it is found that authors have tended to relate the concentration of potassium to the level of nitrate. This is correlated with a coefficient of 0.78, $P<0.001$ (George *et al.,* 1988). The average concentration of potassium in these media was 13.6 mM and the most common value (median), 10.5 mM. Murashige and Skoog (1962) medium contains 20.04 mM K^+.

2.4. SODIUM

Sodium ions (Na^+) are taken up into plants, but in most cases they are not required for growth and development and many plants actively secrete them from their roots to maintain a low internal concentration. The element can function as an osmotic stabilizer in halophytic plants; these have become adapted so that, in saline soils with low water potential, they can accumulate abnormally high concentrations of Na^+ ion in vacuoles, and thereby maintain sufficient turgor for growth.

Sodium does appear to have a beneficial nutritional effect on some plants and is therefore considered as a functional element (Subbarao *et al.,* 2003). Small amounts of sodium chloride (e.g. 230 mg/l) can stimulate the growth of plants in the families Chenopodiaceae and Compositae even when there is no limitation on the availability of K^+ (Brownell, 1979). In other plants such as wheat, oats, cotton and cauliflower (Sharma and Singh, 1990), sodium can partially replace potassium, but is not essential.

Sodium only appears to be essential to those salt-tolerant plants, which have a C4 (crassulacean acid) metabolism. Examples are *Bryophyllum tubiflorum* (Crassulaceae) and *Mesembryanthemum crystallimum* (Aizoaceae). In these plants the element is necessary for CO_2 fixation in photosynthesis.

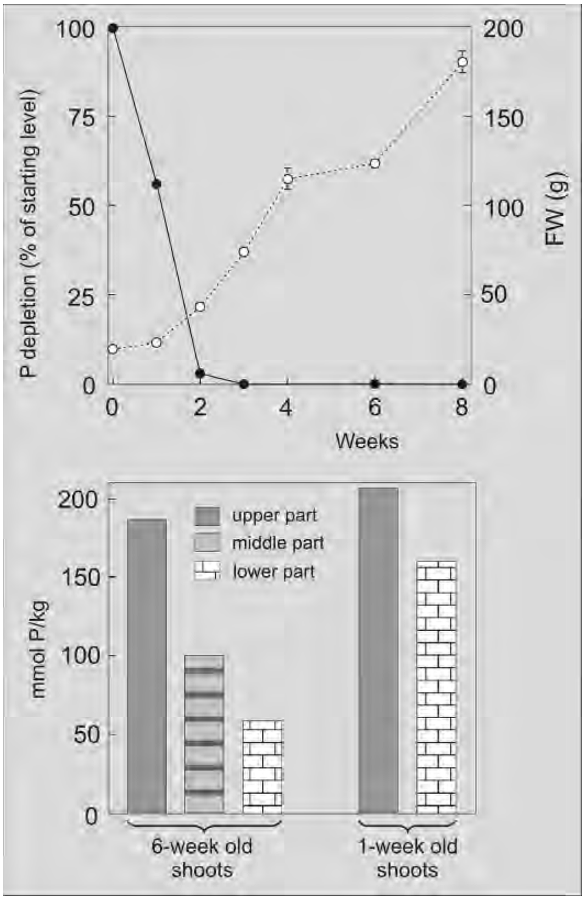

Fig. 3.4 Top: Depletion of P in the medium compared to the growth of *Dahlia* cultures.
Bottom (*Right*) P content in 1-week old *Dahlia* shoots taken from the culture after 1 week when P had not yet been exhausted. (*Left*) 6-week old shoots in which the upper part had been formed after all P had been taken up from the medium. The high content in the newly formed upper part of the shoots indicates massive retranslocation of P after uptake from older to newer tissue (G. de Klerk, unpub. data).

Most macronutrient formulations do not contain any sodium at all, and the average concentration in 615 different preparations was 1.9 mM (George *et al.,* 1988). Even if the element is not deliberately added as a macronutrient, small amounts are incorporated in most media from the salts added to provide micronutrients. Plant macronutrient preparations

containing high concentrations of both sodium and chloride ions are not well formulated.

2.5. MAGNESIUM

Magnesium is an essential component of the chlorophyll molecule and is also required non-specifically for the activity of many enzymes, especially those involved in the transfer of phosphate. ATP synthesis has an absolute requirement for magnesium and it is a bridging element in the aggregation of ribosome subunits. Magnesium is the central atom in the porphyrin structure of the chlorophyll molecule. Within plants, the magnesium ion is mobile and diffuses freely and thus, like potassium, serves as a cation balancing and neutralising anions and organic acids. Macklon and Sim (1976) estimated there to be 2.1 mM Mg^{2+} in the cytoplasm of *Allium cepa* roots while McClendon (1976) put the general cytoplasmic requirement of plants as high as 16 mM. Plant culture media invariably contain relatively low concentrations of magnesium (average 6.8 mM, median 5.3 mM). Very often $MgSO_4$ is used as the unique source of both magnesium and sulphate ions.

Walker and Sato (1981) found there to be a large reduction in the number of somatic embryos formed from *Medicago sativa* callus when Mg^{2+} was omitted from the medium. In sympathy with this finding, Kintzios *et al.,* (2004) observed in tissue culture of melon that the highest level of magnesium occurred in direct somatic embryogenic cultures and the lowest level in callus cultures.

2.6. SULPHUR

The sulphur utilised by plants is mainly absorbed as SO_4^{2-}, which is the usual source of the element in plant culture media. Uptake is coupled to nitrogen assimilation (Reuveny *et al.,* 1980), and is said to be independent of pH. It results in the excretion of OH^- ions by the plant, making the medium more alkaline. However, according to Mengel and Kirkby (1982), plants are relatively insensitive to high sulphate levels and only when the concentration is in the region of 50 mM, is growth adversely affected. Although sulphur is mainly absorbed by plants in the oxidized form, that which is incorporated into chemical compounds is mainly as reduced -SH, -S- or -S-S- groups. The sulphur-containing amino acids cysteine and methionine become incorporated into proteins. Sulphur is used by plants in lipid synthesis and in regulating the structure of proteins through the formation of S-S bridges. The element also acts as a ligand joining ions of iron, zinc and copper to metalloproteins and enzymes. The reactive sites of some enzymes are -SH groups. Sulphur is therefore an essential element and deficiency results in a lack of protein synthesis. Sulphur-deficient plants are rigid, brittle and thin-stemmed. Important sulphur compounds are glutathione, which acts in detoxification of oxygen radicals, and the proteins thioredoxin and ferrodoxin that are involved in redox chemistry.

Growth and protein synthesis in tobacco cell suspensions were reduced on a medium containing only 0.6 mM SO_4^{2-} instead of 1.73 mM (Klapheck *et al.,* 1982) and when the supply of S in the medium was used up, large amounts of soluble nitrogen accumulated in the cells. Most media contain from 2-5 meq/l SO_4^{2-} (1 – 2.5 mM).

2.7. CALCIUM

As a major cation, calcium helps to balance anions within the plant, but unlike potassium and magnesium, it is not readily mobile. Because of its capacity to link biological molecules together with coordinate bonds, the element is involved in the structure and physiological properties of cell membranes and the middle lamella of cell walls. The enzyme β-(1→3)-glucan synthase depends on calcium ions, and cellulose synthesis by cultured cells does not occur unless there are at least micro-molar quantities of Ca^{2+} in the medium. Many other plant enzymes are also calcium-dependent and calcium is a cofactor in the enzymes responsible for the hydrolysis of ATP.

Although calcium can be present in millimolar concentrations within the plant as a whole, calcium ions are pumped out of the cytoplasm of cells. Ca^{2+} is sequestered in the vacuole, complexes with calcium-binding proteins and may precipitate into calcium oxalate crystals to maintain the concentration at around only 0.1 mM. The active removal of Ca^{2+} is necessary to prevent the precipitation of phosphate (and the consequent disruption of phosphate-dependent metabolism) and interference with the function of Mg^{2+}. The uniquely low intra-cellular concentration of Ca^{2+} allows plants to use calcium as a chemical 'second messenger' (Hepler and Wayne, 1985; Sanders *et al.,* 1999). Regulatory mechanisms are initiated when Ca^{2+} binds with the protein calmodulin, which is thus enabled to modify enzyme activities. A temporary increase in Ca^{2+}concentration to 1 or 10 mM does not significantly alter the ionic environment within the cell, but is yet sufficient to

trigger fundamental cell processes such as polarized growth (for example that of embryos - Shelton *et al.,* 1981), response to gravity and plant growth substances, cytoplasmic streaming, and mitosis (Ferguson and Drbak, 1988; Poovaiah, 1988). Physiological and developmental processes, which are initiated through the action of phytochrome are also dependent on the presence of Ca^{2+} (Shacklock *et al.,* 1992). A short-term increase in cytosolic free Ca^{2+} has been observed for osmoadaptation (Taylor *et al.,* 1996), phytoalexin synthesis (Knight *et al.,* 1991), thermotolerance (Gong *et al.,* 1998) and induction of free-radical scavengers (Price *et al.,* 1994).

Large quantities of calcium can be deposited outside the protoplast, in cell vacuoles and in cell walls. Calcification strengthens plant cell walls and is thought to increase the resistance of a plant to infection. By forming insoluble salts with organic acids, calcium immobilises some potentially damaging by-products. The element gives protection against the effects of heavy metals and conveys some resistance to excessively saline conditions and low pH.

The Ca^{2+} ion is involved in *in vitro* morphogenesis and is required for many of the responses induced by plant growth substances, particularly auxins and cytokinins. In the moss *Funaria*, cytokinin causes an increase in membrane-associated Ca^{2+} specifically in those areas which are undergoing differentiation to become a bud (Saunders and Hepler, 1981). Protocorm formation from callus of *Dendrobium fibriatum* was poor on Mitra *et al.,* (1976) A medium when calcium was omitted (Mitra *et al.,* 1976) and in *Torenia* stem segments, adventitious bud formation induced by cytokinin seems to be mediated, at least in part, by an increase in the level of Ca^{2+} within cells (Tanimoto and Harada, 1986). Exogenous Ca^{2+} enhanced the formation of meristemoids and the first phases of outgrowth into organs in tobacco pith explants (Capitani and Altamura, 2004). In carrot, somatic embryogenesis coincides with a rise of free cytosolic Ca^{2+} (Timmers *et al.,* 1996) and applied Ca^{2+} increases the number of somatic embryos (Jansen *et al.,* 1990).

2.7.1. Shoot tip necrosis

Calcium deficiency in plants results in poor root growth and in the blackening and curling of the margins of apical leaves, often followed by a cessation of growth and death of the shoot tip. The latter symptoms are similar to aluminium toxicity (Wyn Jones and Hunt, 1967). Tip necrosis has been especially observed in shoot cultures, sometimes associated with hyperhydricity. It often occurs after several subcultures have been accomplished (e.g. in *Cercis canadensis* - Yusnita *et al.,* 1990). After death of the tip, shoots often produce lateral branches, and in extreme cases the tips of these will also die and branch again. The cause of tip necrosis has not always been determined [e.g. in *Pistacia* shoot cultures (Barghchi, 1986), where shoots showing symptoms may die after planting out (Martinelli, 1988)]. The occurrence of necrosis was reduced in *Pistacia* (Barghchi *loc. cit.*) and *Prunus tenella* (Alderson *et al.,* 1987) by more frequent subculturing, but this is a costly and time-consuming practice. In *Pictacia*, calcium reduced necrosis (Barghchi and Alderson, 1996).

Tip necrosis was found in *Psidium guajava* shoot cultures after prolonged subculturing, if shoots were allowed to grow longer than 3 cm, and was common in rapidly growing cultures (Amin and Jaiswal, 1988); it occurred on *Sequoiadendron giganteum* shoots only when they were grown on relatively dilute media (Monteuuis *et al.,* 1987). Necrosis of *Rosa hybrida* 'White Dream', was cured by adding 0.1 mg/l GA_3 to the medium (Valle and Boxus, 1987).

Analysis of necrotic apices has shown them to be deficient in calcium (Debergh, 1988), and a shortage of this element has been associated with tip necrosis in *Amelanchier*, *Betula*, *Populus*, *Sequoia*, *Ulmus, Cydonia* and other woody plants, although the extent of damage is variable even between genotypes within a species (Sha *et al.,* 1985; Singha *et al.,* 1990). As calcium is not remobilised within plant tissues, actively growing shoots need a constant fresh supply of ions in the transpiration stream. An inadequate supply of calcium can result from limited uptake of the ion, and inadequate transport, the latter being caused by the absence of transpiration due to the high humidity in the culture vessel. A remedy can sometimes be obtained by reducing the culture temperature so that the rate of shoot growth matches calcium supply, using vessels which promote better gas exchange (thereby increasing the transpiration and xylem transport), or by increasing the concentration of calcium in the medium (McCown and Sellmar, 1987). The last two remedies can have drawbacks: the medium will dry out if there is too free gas exchange; adding extra calcium ions to the medium is not always effective (e.g. in cultures of *Castanea sativa* - Mullins, 1987); and can introduce

undesirable anions. Chloride toxicity can result if too much calcium chloride is added to the medium (see below). To solve this difficulty, McCown *et al.,* (Zeldin and McCown, 1986; Russell and McCown, 1988) added 6 mM calcium gluconate to Lloyd and McCown (1981) WPM medium to correct Ca^{2+} deficiency, without altering the concentrations of the customary anions. There is a limit to the concentration of calcium, which can be employed in tissue culture media because several of its salts have limited solubility.

2.8. CHLORIDE

The chloride ion (Cl^-) has been found to be essential for plant growth (Broyer *et al.,* 1954; Johnson *et al.,* 1957; Ozanne *et al.,* 1957; Ozanne, 1958), but seems to be involved in few biological reactions and only very small quantities are really necessary. Rains (1976) listed chlorine as a micronutrient. Chloride is required for the water-splitting protein complex of Photosystem II, and it can function in osmoregulation in particular in stomatal guard cells. The chloride ion is freely transported and many plants can tolerate the presence of high concentrations without showing toxicity. The chief role of chloride seems to be in the maintenance of turgor and in balancing rapid changes in the level of free cations such as K^+, Mg^{2+} and Na^+. Plants deprived of Cl^- are liable to wilting (Johnson *et al.,* 1957).

In isolated chloroplasts, chloride (together with Mn^{2+}) ions are required for oxygen evolution in photosystem II of photosynthesis (Bov *et al.,* 1963; Mengel and Kirkby, 1982; Shkolnik, 1984), although there has been some doubt whether this requirement exists *in vivo* (Terry, 1977). Chloride ions are best taken into plants at slightly acid pH (Jacobson *et al.,* 1971).

The most common concentration of chloride in culture media is 3 mM, the average 6 mM. MS medium contains 6 mM Cl^-; Quoirin and Lepoivre (1977) medium, only 0.123 μM. Some species are sensitive to chloride ions. McCown and Sellmer (1987) reported that too high a concentration, seemed to cause woody species to have yellow leaves and weak stems: sometimes tissues collapsed and died. An excess of Cl^- has been thought to be one cause of the induction of hyperhydricity, and omission of the ion does seem to prevent the development of these symptoms in *Prunus* (Volume 2). Pevalek-Kozlina and Jelaska (1987) deliberately omitted chloride ions from WPM medium for the shoot culture of *Prunus avium* and obtained infrequent hyperhydricity in only one genotype. The presence of 7 mM Cl^- can be toxic to pine suspension cultures (Teasdale, 1987).

As chlorine has only a relatively small nutritional significance, steps are sometimes taken to reduce the concentration of chloride ion in culture media, but in order to adjust the concentration of other ions, it is then often necessary to make a marked increase in SO_4^{2-}. For example, using ammonium sulphate instead of ammonium chloride to supply NH_4^+ in Eeuwens (1976) Y3 medium, would increase the sulphate level from 2 to 12 meq/l (from 1 to 6 mM).

3. MICRONUTRIENTS

Plant requirements for microelements have only been elucidated over the past 50-60 years. Before the end of the last century, it had been realised that too little iron caused chlorophyll deficiency in plants, but the importance of other elements took many years to prove conclusively. Mazé, for example, used hydroponic techniques during the years 1914-1919 to show that zinc, manganese and boron improved the growth of maize plants. Sommer and Lipman (1926) also showed the essentiality of boron, and Sommer (1931) of copper, but uncertainty over which elements were really indispensible to growth still existed in 1933 when Hoagland and Snyder proposed two supplementary nutrient solutions for water culture which in total contained 26 elements. It took several further years to prove that molybdenum (Arnon and Stout, 1939) and cobalt in very small amounts, were most important for healthy plant growth. Early plant tissue culture work was to both profit from, and contribute to the findings of previous hydroponic studies. Our understanding has been enhanced by investigations into the biochemical role of minor elements.

3.1. EARLY USE IN PLANT TISSUE CULTURE MEDIA

At the time of the early plant tissue culture experiments, uncertainty still existed over the nature of the essential microelements. Many tissues were undoubtedly grown successfully because they were cultured on media prepared from impure chemicals (see below) or solidified with agar, which acted as a micronutrient source.

In the first instance, the advantage of adding various micronutrients to culture media was mainly evaluated by the capability of individual elements to improve the growth of undifferentiated callus or isolated root cultures. Knudson (1922) incorporated Fe and Mn in his very successful media for the non-symbiotic germination of orchid seeds, and, following a recommendation by Berthelot (1934), Gautheret (1939) and Nobécourt (1937) included in their media (in addition to iron) copper, cobalt, nickel, titanium and beryllium. Zinc was found to be necessary for the normal development of tomato root systems (Eltinge and Reed, 1940), and without Cu, roots ceased to grow (Glasstone, 1947). Hannay and Street (1954) showed that Mo and Mn were also essential for root growth.

An advantage adding five micronutrients to tissue culture media was perhaps first well demonstrated by Heller in 1953 who found that carrot callus could be maintained for an increased number of passages when Fe, B, Mn, Zn and Cu were present.

3.2. MICRONUTRIENTS FROM TRACE IMPURITIES

Micronutrients tend to be added to modern media by the addition of fairly standard chemicals. Street (1977) rightly emphasised that even analytical grade chemicals contain traces of impurities that will provide a hidden supply of micronutrients to a medium. An illustration of this, comes from the work of Dalton *et al.,* (1983) who found traces of silicon (Si) in a precipitate from MS medium which had been made up with analytical grade laboratory chemicals. Gelling agents contain inorganic elements but whether cultures can utilize them is unclear. Amounts of contaminating substances in chemicals would have been greater in times past, so that an early medium such as Knudson (1922; 1943) B, prepared today with highly purified chemicals, will not have quite the same composition as when it was first used by Knudson in 1922; the addition of some micronutrients might improve the results obtained from a present-day formulation of such early media.

3.3. OPTIMUM MICRO-ELEMENT CONCENTRATIONS

Most modern culture media use the microelements of Gamborg *et al.,* (1968) B5 medium, or the more concentrated mixtures in MS or Bourgin and Nitsch (1967) H media. Several research workers have continued to use Heller (1953) micro-nutrient formulation, even though higher levels are now normally recommended. Quoirin and Lepoivre (1977) showed clearly that in conjunction with MS or their Quoirin and Lepoivre (1977) B macro-elements, the concentration of Mn in Heller's salts should be increased by 100-fold to obtain the most effective growth of *Prunus* meristems.

Cell growth and morphogenesis of some species may even be promoted by increasing the level of micronutrients above that recommended by Murashige and Skoog (1962). The induction and maintenance of callus and growth of cell suspensions of juvenile and mature organs of both Douglas fir and loblolly pine, was said to be improved on Litvay *et al.,* (1981) LM medium in which Mg, B, Zn, Mo, Co and I were at 5 times the concentration of MS micro-elements, and Mn and Cu at 1.25 and 20 times respectively (Litvay *et al.,* 1981; Verma *et al.,* 1982). Other authors to have employed high micronutrient levels are Barwale *et al.,* (1986) who found that the induction of adventitious shoots from callus of 54 genotypes of *Glycine max* was assisted by adding four times the normal concentration of minor salts to MS medium.

A further example of where more concentrated micro-elements seemed to promote morphogenesis is provided by the work of Wang, *et al.,* (1980, 1981). Embryogenesis could be induced most effectively in callus derived from *Hevea brasiliensis* anthers, by doubling the concentration of microelements in MS medium, while at the same time reducing the level of macronutrients to 60-80% of the original.

Despite these reports, few research workers seem to have accepted the need for such high micronutrient levels. To diminish the occurrence of hyperhydricity in shoot culture of carnation, Dencso (1987) reduced the level of micronutrients (except iron, which was as recommended by Dalton *et al.,* 1983) to those in MS medium, but this mixture was inadequate for *Gerbera* shoot cultures and the rate of propagation was less than that with the normal MS formulation.

The need for macronutrient concentrations to be optimised as the first step in media development seems to be emphasised by results of Eeuwens (1976). In an experiment with factorial combinations of the macro- and micro-nutrient components of four media, his Eeuwens (1976) Y3 micronutrients gave a considerable improvement in the growth of coconut callus, compared with other micro-element mixtures, when they were used with Y3 and MS macronutrients, but not when used with those of White (1942) or Heller (1953; 1955).

3.4. CELLULAR DIFFERENTIATION AND MORPHOGENESIS

Welander (1977) obtained evidence, which suggested that plant cells are more demanding for minor elements when undergoing morphogenesis. Petiole explants of *Begonia hiemalis* produced callus on media without micronutrients, but would only produce adventitious shoot buds directly when micronutrients were added to the macronutrient formulation. The presence of iron is particularly important for adventitious shoot and root formation (Legrand, 1975).

That mineral nutrition can influence cellular differentiation in combination with growth hormones, was shown by Beasley *et al.,* (1974). Cotton ovules cultured on a basic medium containing 5.0 mM IAA and 0.5 mM GA_3, required 2 mM calcium (normally present in the medium) for the ovules to develop fibres. Magnesium and boron were essential for fibre elongation.

3.5. THE ROLES OF MICRONUTRIENTS

3.5.1. Manganese

The essential micronutrient metals Fe, Mn, Zn, B, Cu, Co and Mo are components of plant cell proteins of metabolic and physiological importance. At least five of these elements are, for instance, necessary for chlorophyll synthesis and chloroplast function (Sundqvist *et al.,* 1980). Micronutrients have roles in the functioning of the genetic apparatus and several are involved with the activity of growth substances.

Manganese (Mn) is one of the most important microelements and has been included in the majority of plant tissue culture media. It is generally added in similar concentrations to those of iron and boron, i.e. between 25-150 mM. Manganese has similar chemical properties to Mg^{2+} and is apparently able to replace magnesium in some enzyme systems (Hewitt, 1948). However there is normally 50- to 100-fold more Mg^{2+} than Mn^{2+} within plant tissues, and so it is unlikely that there is frequent substitution between the two elements.

The most probable role for Mn is in definition of the structure of metalloproteins involved in respiration and photosynthesis (Clarkson and Hanson, 1980). It is known to be required for the activity of several enzymes, which include decarboxylases, dehydrogenases, kinases and oxidases and superoxide dismutase enzymes. Manganese is necessary for the maintenance of chloroplast ultra-structure. Because Mn(II) can be oxidized to Mn(IV), manganese plays an important role in redox reactions. The evolution of oxygen during photosystem II of the photosynthetic process, is dependent on a Mn-containing enzyme and is proportional to Mn content (Mengel and Kirkby, 1982; Shkolnik, 1984). Mn is toxic at high concentration (Sarkar *et al.,* 2004).

In tissue cultures, omission of Mn ions from Doerschug and Miller (1967) medium reduced the number of buds initiated on lettuce cotyledons. A high level of manganese could compensate for the lack of molybdenum in the growth of excised tomato roots (and vice versa) (Hannay and Street, 1954). Natural auxin levels are thought to be reduced in the presence of Mn^{2+} because the activity of IAA-oxidase is increased. This is possibly due to Mn^{2+} or Mn-containing enzymes inactivating oxidase inhibitors, or because manganous ions are one of the cofactors for IAA oxidases in plant cells (Galston and Hillman, 1961). Manganese complexed with EDTA increased the oxidation of naturally-occurring IAA, but not the synthetic auxins NAA or 2,4-D (MacLachlan and Waygood, 1956). However, Chée (1986) has suggested that, at least in blue light, Mn^{2+} tends to cause the maintenance of, or increase in, IAA levels within tissues by inactivating a co-factor of IAA oxidase. When the Mn^{2+} level in MS medium was reduced from 100 mM to 5 mM, the production in blue light, of axillary shoots by *Vitis* shoot cultures was increased.

3.5.2. Zinc

Zinc is a component of stable metallo-enzymes with many diverse functions, making it difficult to predict the unifying chemical property of the element, which is responsible for its essentiality (Clarkson and Hanson, 1980). Zinc is required in more than 300 enzymes including alcohol dehydrogenase, carbonic anhydrase, superoxide dismutase and RNA-polymerase. Zinc forms tetrahedral complexes with N-, O-, and S-ligands. In bacteria, Zn is present in RNA and DNA polymerase enzymes, deficiency resulting in a sharp decrease in RNA levels. DNA polymerase is concerned with the repair of incorrectly formed pieces of DNA in DNA replication, and RNA polymerase locates the point on the DNA genome at which initiation of RNA synthesis is to take place. Divalent Mg^{2+}, Mn^{2+} or Co^{2+} are also required for activation of these enzymes (Eichhorn, 1980).

Zinc deficient plants suffer from reduced enzyme activities and a consequent diminution in protein, nucleic acid and chlorphyll synthesis. Molybdenum- and zinc-deficient plants have a decreased chlorophyll content and poorly developed

chloroplasts. Plants deprived of zinc often have short internodes and small leaves.

The concentration of Zn^{2+} in MS medium is 30 µM but amounts added to culture media have often varied widely between 0.1-70 µM and experimental results to demonstrate the most appropriate level are limited. When Eriksson (1965) added 15 mg/l $Na_2ZnEDTA.2H_2O$ (40µM Zn^{2+})to *Haplopappus gracilis* cell cultures, he obtained a 15% increase in cell dry weight which was thought to be due to the presence of zinc rather than the chelating agent. Zinc was also shown to increase growth of a rice suspension. The highest concentration tested, 520 µM, resulted in the fastest rate of growth and it was suggested that zinc had increased auxin activity (see below) (Hossain *et al.,* 1997). Zinc is required for adventitious root formation in *Eucalyptus* (Schwambach *et al.,* 2005). In cassava, additional zinc promotes somatic embryogenesis and rooting (C.J.J.M Raemakers, pers. commun.). However, very high concentrations of zinc are found to be inhibitory, and the microelement has been noted to prevent root growth at a concentration higher than 50 µM.

There is a close relationship between the zinc nutrition of plants and their auxin content (Skoog, 1940). It has been suggested that zinc is a component of an enzyme concerned with the synthesis of the IAA precursor, tryptophan (Tsui, 1948). The importance of Zn for tryptophan synthesis is especially noticeable in crown gall callus which normally produces sufficient endogenous auxin to maintain growth on a medium without synthetic auxins, but which becomes auxin-deficient and ceases to grow in the absence of Zn (Klein *et al.,* 1962).

3.5.3. Boron

Boron is involved in plasma membrane integrity and functioning, probably by influencing membrane proteins, and cell wall intactness. Reviews have been provided by Lewis (1980) and by Blevins and Lukaszewski (1998). The element is required for the metabolism of phenolic acids, and for lignin biosynthesis: it is probably a component, or co-factor of the enzyme which converts *p*-coumaric acid to caffeate and 5-hydroxyferulate. Boron is necessary for the maintenance of meristematic activity, most likely because it is involved in the synthesis of N-bases (uracil in particular); these are required for RNA synthesis (Mengel and Kirkby, 1982). It is also thought to be involved in the maintenance of membrane structure and function, possibly by stabilizing natural metal chelates which are important in wall and membrane structure and function (Pollard *et al.,* 1977; Clarkson and Hanson, 1980). Boron is concerned with regulating the activities of phenolase enzymes; these bring about the biosynthesis of phenylpropane compounds, which are polymerized to form lignin. Lignin biosynthesis does not take place in the absence of boron. Boron also mediates the action of phytochrome and the response of plants to gravity (Tanada, 1978).

Use in culture media. In the soil, boron occurs in the form of boric acid and it is this compound, which is generally employed as the source of the element in tissue cultures. Uptake of boric acid occurs most readily at acid pH, possibly in the undissociated form (Oertli and Grgurevic, 1974) or as $H_2BO_3^-$ (Devlin, 1975). A wide range of boron concentrations has been used in media, the most usual being between from 50 and 100 µM: MS medium contains 100 µM. Bowen (1979) found boron to be toxic to sugarcane suspensions above 2 mg/l (185 µM), but there are a few reports of higher concentrations being employed (Table 3.8). High concentrations of boron may have a regulatory function; for example, 1.6-6.5 mM have been used in simple media to stimulate pollen germination (Brewbaker and Kwack, 1963; Taylor, 1972).

Boric acid reacts with some organic compounds having two adjacent *cis*-hydroxyl groups (Greenwood, 1973). This includes *o*-diphenols, hexahydric alcohols such as mannitol and sorbitol (commonly used in plant tissue culture as osmotic agents), and several other sugars, but excludes sucrose which forms only a weak association. Once the element is complexed it appears to be unavailable to plants. This led Lewis (1980) to suggest that because boric acid was required for lignin biosynthesis, vascular plants were led, during evolution, to use sucrose exclusively for the transport of carbohydrate reserves.

Although the addition of sugar alcohols and alternative sugars to sucrose can be beneficial during plant tissue culture (see Chapter 4), it is clearly necessary to return to a sucrose-based medium for long-term culture, or boron deficiency may result.

Deficiency symptoms. Boron is thought to promote the destruction of natural auxin and increase its translocation. Endogenous IAA levels increase in the absence of boron and translocation is reduced, the compound probably being retained at the site of synthesis (Goldbach and Amberger, 1986). Plants suffering from boron deficiency have restricted root systems (Odhnoff, 1957; Whittington, 1959) and a

reduced capacity to absorb $H_2PO_4^-$ and some other ions. High levels of auxins can have the same effect on growth and ion uptake (Pollard *et al.,* 1977). Neales (1959, 1964) showed that isolated roots stopped growing unless a minimum concentration of boron was present (although the necessity for the element was not so apparent when cultures were grown in borosilicate glass vessels). Inhibition of root elongation in the absence of boron has been shown to be due to the cessation of mitosis and the inhibition of DNA synthesis (Moore and Hirsch, 1981). Boron deficiency also results in depressed cytokinin synthesis. Cell division is inhibited in the absence of boron, apparently because there is a decrease in nuclear RNA synthesis (Ali and Jarvis, 1988). However, deficiency often leads to increased cambial growth in intact dicotyledonous plants.

Table 3.8 Examples of cultures grown with unusually high concentrations of boron

Plant	Concentration of boron (μM)	Type of Culture	Reference
Antirrhinum majus	323	Embryos from protoplast colonies	Poirier-Hamon *et al.,* (1974)
Brassica napus *Capsella bursa-pastoris*	200	Embryo	Monnier (1976)
Citrus medica	646	Anther callus	Drira and Benbadis (1975)
Hevea brasiliensis	320	Anther: plant regeneration	Chen (1984)
Hordeum crosses	242	Embryo rescue	Jensen (1974)
Larix deciduas	250	Direct morphogenesis	Bonga (1984))
	250	Callus and embryogenesis	Nagmani and Bonga (1985)
Lycopersicon esculentum	242	Isolated root	Street and McGregor (1952)
Nicotiana tabacum	323	Protoplant culture	Ohyama and Nitsch (1972)
Petunia hybrida	566	Callus and root formation	Sangwan and Norreel (1975)
Prunus amygdalus	200	Shoot	Hisjima (1982a)

One of the changes seen in some plants grown under boron deficiency is the outgrowth of lateral buds resulting in plants with a bushy or rosette appearance. In pea, this was associated with a sharp decrease in IAA-export from the apex (Li *et al.,* 2001). It is generally accepted that the outgrowth of lateral buds is inhibited by polar auxin transport in the stem and that disruption of this transport by decapitation or auxin transport inhibitors results in the outgrowth of lateral buds (Tamas, 1987).

Cotton ovules which otherwise develop fibres when cultured, commence extensive callus formation when placed on a medium deficient in boron. On the other hand, the growth rate of callus cultures of *Helianthus annuus* and *Daucus carota* (Krosing, 1978), and cell cultures of sugar cane (Bowen, 1979), was much reduced when boron was not present in the growth medium. Boron influences the development of the suspensor of somatic embryos in *Larix deciduas* (Behrendt and Zoglauer, 1996). Boron had no influence on the induction of embryogenesis in *Daucus carota* but altered the development of embryos: root development was promoted at low concentrations and shoot development at high. This coincided with a high and low auxin-cytokinin ratio, respectively (Mashayekhi and Neumann, 2006).

Adventitious root formation. Boron is thought to promote the destruction of auxin. Although auxin is required for the formation of adventitious root initials, boron is necessary in light grown-plants for the growth of these primordia (Middleton *et al.,* 1978); possibly boron enhances the destruction of auxin in these circumstances, which in high concentrations is inhibitory to root growth (Jarvis, 1986). An interaction between boron and auxins in the rooting of cuttings has been noticed in several species (Hemberg, 1951; Weiser, 1959; Weiser and Blaney, 1960; Bowen *et al.,* 1975; Josten and Kutschera, 1999) and a supply of exogenous borate has been shown to be essential (Ali and Jarvis, 1988). However, excessive boron concentrations lead to a reduction in the number of roots formed (Jarvis, 1986). Boron deficiency had no observed effect

on the rooting of *Eucalyptus* microcuttings (Schwambach *et al.,* 2005).

3.6. COPPER AND MOLYBDENUM

Copper is an essential micronutrient, even though plants normally contain only a few parts per million of the element. Two kinds of copper ions exist; they are the monovalent cuprous [Cu(I)] ion, and the divalent cupric [Cu(II)] ion: the former is easily oxidized to the latter; the latter is easily reduced. The element becomes attached to enzymes, many of which bind to, and react with oxygen. They include the cytochrome oxidase enzyme system, responsible for oxidative respiration, and superoxide dismutase (an enzyme which contains both copper and zinc atoms). Detrimental superoxide radicals, which are formed from molecular oxygen during electron transfer reactions, are reacted by superoxide dismutase and thereby converted to water. Copper atoms occur in plastocyanin, a pigment participating in electron transfer.

Several copper-dependent enzymes are involved in the oxidation and hydroxylation of phenolic compounds, such as ABA and dopamine (Lerch, 1981). The hydroxylation of monophenols by copper-containing enzymes leads to the construction of important polymeric constituents of plants, such as lignin. These same enzymes can lead to the blackening of freshly isolated explants. Copper is a constituent of ascorbic acid oxidase and the characteristic growth regulatory effects of ethylene are thought to depend on its metabolism by an enzyme, which contains copper atoms.

High concentrations of copper can be toxic. Most culture media include ca. 0.1-1.0 µM Cu^{2+}. Ions are usually added through copper sulphate, although occasionally cupric chloride or cupric nitrate have been employed. In hydroponic culture of *Trifolium pratense*, uptake of copper into the plant depended on the amount of nitrate in solution. Uptake was considerably reduced when NO_3^- was depleted (Jarvis, 1984). The concentration of Cu in tissue culture media is very small relative to the level in plants (Table 3.1). It is therefore not surprising that various authors report strong increases of growth when Cu is added at 1- 5 µM (Dahleen, 1995; Nirwan and Kothari, 2003; Kintzios *et al.,* 2001; Nas and Read, 2004; Bouman and Tiekstra, 2005)

Plants utilise hexavalent molybdenum and absorb the element as the molybdate ion (MoO_4^{2-}). This is normally added to culture media as sodium molybdate at concentrations up to 1 mM. Considerably higher levels have occasionally been introduced [e.g. in the media of Abou-Mandour (1977) and Asahira and Kano (1977)] apparently without adverse effect, although Teasdale (1987) found pine suspension cultures were injured by 50 mM. Molybdenum is a component of several plant enzymes, two being nitrate reductase and nitrogenase, in which it is a cofactor together with iron: it is therefore essential for nitrogen utilisation. Tissues and organs presented with NO_3^- in a molybdenum-deficient medium can show symptoms of nitrate toxicity because the ion is not reduced to ammonia.

3.7. COBALT

Cobalt is not regarded as an essential element. Nevertheless, it was found to have been included in approximately half of a large sample of published plant culture media (George, *et al.,* 1987). Murashige and Skoog (1962) included Co in their medium because it had been shown to be required by lower plants (Holm-Hansen *et al.,* 1954) and that it might have a role in regulating morphogenesis in higher plants (Miller, 1954; Salisbury, 1959). However, no stimulatory effect on the growth of tobacco callus was observed by adding cobalt chloride to the medium at several concentrations from 0.1 µM and above, and at 80.0 and 160 µM the compound was toxic. Similarly Schenk and Hildebrandt (1972) obtained no clear evidence for a Co requirement in tests on a wide variety of plants, but retained the element in their medium because they occasionally observed an apparent stimulation to the callus growth of some monocotyledons. *Pinus* suspension cultures do not require cobalt (Teasdale, 1987). The concentration most commonly added to a medium is ca. 0.1 µM, although ten times this amount has sometimes been used. Cobalt is the metal component of Vitamin B12 which is concerned with nucleic acid synthesis (Fries, 1962), but evidence that the element has any marked stimulatory effect on growth or morphogenesis in plant tissue cultures is hard to find. Cobalt may replace nickel in urease and thereby render it inactive, e.g., in potato (Witte *et al.,* 2002).

Advantage from adding cobalt to plant culture media might be derived from the fact that the element can have a protective action against metal chelate toxicity and it is able to inhibit oxidative reactions catalysed by ions of copper and iron (Albert, 1958). The Co^{2+} ion can inhibit ethylene synthesis.

3.8. ALUMINIUM AND NICKEL

Several workers, following Heller (1953), have included aluminium and nickel in their micronutrient formulations. However, the general benefit of adding the former metal does not seem to have been adequately demonstrated.

It was believed that in most plants Ni^{2+} is not absolutely required for normal growth and development (Mishra and Kar, 1975). However, more recently, it has been found by careful experimentation that nickel is essential (Gerendás *et al.,* 1999). The ion is a component of urease enzymes (Dixon *et al.,* 1975; Polacco, 1977a), which convert urea to ammonia. It has been shown to be an essential micronutrient for some legumes and to actvate urease in potato microshoot cultures (Witte et al., 2002). In tissue cultures the presence of 0.1 mM Ni^{2+} strongly stimulates the growth of soybean cells in a medium containing only urea as a nitrogen source. Slow growth occurs on urea without the deliberate addition of nickel, possibly supported by trace amounts of the element remaining in the cells (Polacco, 1977b). Cells and tissues are not normally grown with urea as a nitrogen source, and as urease is the only enzyme, which has been shown to have a nickel component, it could be argued that nickel is not essential. However, without it soybean plants grown hydroponically, accumulate toxic concentrations of urea (2.5%) in necrotic lesions on their leaf tips, whether supplied with inorganic nitrogen, or with nitrogen compounds obtained from bacterial symbiotic nitrogen fixation. These symptoms can be alleviated in plants growing in hydroponic culture by adding 1 mg/l Ni to the nutrient solution. Absence of nickel in a hydroponic solution also results in reduced early growth and delayed nodulation (Eskew *et al.,* 1983).

Despite these findings nickel has not been added deliberately to tissue culture media. However, it should be noted that agar contains relatively high levels of nickel (Table 3.2) and the possibility of urea toxicity may have been avoided because, in tissue cultures, urea diffuses into the medium (Teasdale, 1987). Quoirin and Lepoivre (1977) showed that at the concentrations recommended by Heller, Al^{3+} and Ni^{2+} were without effect on the growth of *Prunus* meristems and were inhibitory at higher levels. If it is thought that Ni should be added to a culture medium, 0.1 mM is probably sufficient.

Aluminium has been said to be necessary for the growth of some ferns (Taubck, 1942), but is not generally added to tissue culture media for fern propagation.

3.9. IODINE

Iodine is not recognised as an essential element for the nutrition of plants (Rains, 1976), although it may be necessary for the growth of some algae, and small amounts do accumulate in higher plants (ca.12 and 3 mol/kg dry weight in terrestrial and aquatic plants respectively – Raven, 1986). However, the iodide ion has been added to many tissue culture media (e.g. to 65% of micronutrient formulations).

The practice of including iodide in plant culture media began with the report by White (1938) that it improved the growth of tomato roots cultured *in vitro*. Hannay (1956) obtained similar results and found that root growth declined in the absence of iodine which could be supplied not only from potassium iodide, but also from iodoacetate or methylene iodide, compounds which would only provide iodide ions very slowly in solution by hydrolysis. Street (1966) thought that these results indicated that iodine could be an essential nutrient element, but an alternative hypothesis is that any beneficial effect may result from the ability of iodide ions to act as a reducing agent (George *et al.,* 1988). Oxidants convert iodide ions to free iodine. Eeuwens (1976) introduced potassium iodide into his Y3 medium at 0.05 mM (ten times the level used by Murashige and Skoog), as it prevented the browning of coconut palm tissue cultures. The presence of 0.06 μM potassium iodide slightly improved the survival and growth of cultured *Prunus* meristems (Quoirin and Lepoivre, 1977).

Although Gautheret (1942) and White (1943) had recommended the addition of iodine to media for callus culture, Hildebrandt *et al.,* (1946) obtained no statistically significant benefit from adding potassium iodide to tumour callus cultures of tobacco and sunflower. However, as the average weight of tobacco callus was 11% less without it, the compound was included (at different levels) in both of the media they devised. Once again iodine also had no appreciable effect on tobacco callus yield in the experiments of Murashige and Skoog (1962), but was nevertheless included in their final medium. Other workers have omitted iodine from MS medium (e.g. Roest and Bokelmann, 1975; Périnet *et al.,* 1988; Gamborg, 1991) or from new media formulations without any apparent ill effects. However, Teasdale *et al.,* (1986); Teasdale, (1987) reported a definite requirement of *Pinus taeda* suspensions for 25 mM

KI when they were grown on Litvay *et al.,* (1981) LM medium.

There seems, at least in some plants, to be an interaction between iodine and light. Eriksson (1965) left KI out of his modification of MS medium, finding that it was toxic to *Haplopappus gracilis* cells cultured in darkness: shoot production in *Vitis* shoot cultures kept in blue light was reduced when iodine was present in the medium (Chée, 1986), but the growth of roots on rooted shoots was increased. Chée thought that these results supported the hypothesis that iodine enhanced the destruction and/or the lateral transport of IAA auxin. This seems to be inconsistent with the suggestion that I^- acts mainly as a reducing agent.

3.10. SILICON

Silicon (Si) is the second most abundant element on the surface of the earth. Si has been demonstrated to be beneficial for the growth of plants and to alleviate biotic and abiotic stress (Epstein, 1971). The silicate ion is not normally added to tissue culture media, although it is likely to be present in low concentrations. Deliberate addition to the medium might, however, improve the growth of some plants. Adatia and Besford (1986) found that cucumber plants depleted silicate from a hydroponic solution and in consequence their leaves were more rigid, had a higher fresh weight per unit area and a higher chlorophyll content than the controls. The resistance of the plants to powdery mildew was also much increased.

3.11. IRON

Chelating agents. Some organic compounds are capable of forming complexes with metal cations, in which the metal is held with fairly tight chemical bonds. The complexes formed may be linear or ring-shaped, in which case the complex is called a chelate (from the Greek word meaning a crab's claw). Metals can be bound (or sequestered) by a chelating agent and held in solution under conditions where free ions would react with anions to form insoluble compounds, and some complexes can be more chemically reactive than the metals themselves. For example, Cu^{2+} complexed with amino acids is more active biologically than the free ion (Cruickshank *et al.,* 1987). Chelating agents vary in their sequestering capacity (or avidity) according to chemical structure and their degree of ionisation, which changes with the pH of the solution. Copper is chelated by amino acids at relatively high pH, but in conditions of greater acidity, it is more liable to be complexed with organic acid ligands (White *et al.,* 1981). The higher the stability of a complex, the higher the avidity of the complexing agent. One, and in many cases, two or three molecules of a complexing agent may associate with one metal ion, depending on its valency.

Despite tight bonding, there is always an equilibrium between different chelate complexes and between ions in solution. Complexing agents also associate with some metal ions more readily than with others. In general Fe^{3+} (for agents able to complex with trivalent ions) complexes have a higher stability than those of Cu^{2+}, then (in descending order), Ni^{2+}, Al^{3+} (where possible), Zn^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+} and Ca^{2+} (Albert, 1958; Reilley and Schmid, 1958). For a chelated metal ion to be utilised by a plant there must be some mechanism whereby the complex can be broken. This could occur if it is absorbed directly and the ion displaced by another more avid binding agent, or if the complex is biochemically denatured. Metals in very stable complexes can be unavailable to plants; copper in EDTA chelates may be an example (Coombes *et al.,* 1977). High concentrations of avid chelating agents are phytotoxic, probably because they competitively withdraw essential elements from enzymes.

Naturally-occurring compounds act as chelating agents. Within the plant very many constituents such as proteins, peptides, porphyrins, carboxylic acids and amino acids have this property (Albert, 1958; Martin, 1979): some of those with high avidity are metal-containing enzymes. Amino acids are able to complex with divalent metals (Fig. 3.5). Grasses are thought to secrete a chelating agent from their roots to assist the uptake of iron (Römheld and Marschner, 1986). There are also synthetic chelating agents with high avidities (stability constants) for divalent and trivalent ions. Some are listed in Table 3.9, and the structure of those most commonly used in plant culture media is illustrated in Fig 3.6. The application of synthetic chelating agents and chelated micronutrients to the roots of some plants growing in alkaline soils can improve growth by supplying essential metals such as iron and zinc which are otherwise unavailable. The addition of such compounds to tissue culture media can help to make macro- and micro-nutrients more accessible to plant cells.

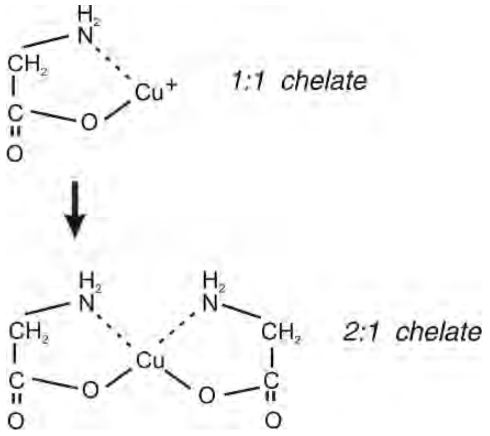

Fig. 3.5 Copper chelated with amino acid, glycine.

Iron chelates. A key property of iron is its capacity to be oxidized easily from the ferrous [Fe(II)] to the ferric [Fe(III)] state, and for ferric compounds to be readily reduced back to the ferrous form. In plants, iron is primarily used in the chloroplasts, mitochondria and peroxisomes of plants for effecting oxidation/reduction (redox) reactions. The element is required for the formation of amino laevulinic acid and protoporphyrinogen (which are respectively early and late precursors of chlorophyll) and deficiency leads to marked leaf chlorosis. Iron is also a component of ferredoxin proteins, which function as electron carriers in photosynthesis.

Iron is therefore an essential micronutrient for plant tissue culture media and can be provided from either ferrous or ferric salts. In early experiments, ferrous sulphate or ferric citrate or tartrate were used in media as a source of the element. Citric and and tartaric acids can act as chelating agents for some divalent metals (Bobtelsky and Jordan, 1945), but are not very efficient at keeping iron in solution (Fig 3.6). If Fe^{2+} and Fe^{3+} ions escape from the chelating agent, they are liable to be precipitated as iron phosphate. The iron may then not be available to plant cells, unless the pH of the medium falls sufficiently to bring free ions back into solution. The problem of precipitation is more severe in aerated media and where the pH of the medium drifts towards alkalinity. Under these conditions Fe^{2+} (ferrous) ions are oxidized to Fe^{3+} (ferric) ions and unchelated ferric ions may then also be converted to insoluble $Fe(OH)_3$. For plant hydroponic culture, the advantages of adding iron to nutrient solutions in the form of a chelate with EDTA was first recognised in the 1950's (Jacobson, 1951; Weinstein *et al.,* 1951). Street *et al.,* (1952) soon found that iron in this form was less toxic and could be utilised by *in vitro* cultures of isolated tomato roots over a wider pH range than ferric citrate. Klein and Manos (1960) showed that callus cultures of several species grew more rapidly on White (1954) medium if Fe^{3+} ions from $Fe_2(SO_4)_3$ were chelated with EDTA, rather than added to the medium from the pure compound, and Doerschug and Miller (1967), that 0.036 mM Fe from NaFeEDTA was as effective as 0.067 mM Fe as ferric citrate, in promoting shoot bud initiation on lettuce cotyledons. Iron presented as ferric sulphate (0.025 mM Fe) was much less effective than either chelated form.

Skoog and co-workers began to use EDTA in media for tobacco callus cultures in 1956 and discussed their findings in the same paper that describes MS medium (Murashige and Skoog, 1962). The addition of an iron (Fe)-EDTA chelate once again greatly improved the availability of the element. Following this publication, (Fe)-EDTA complexes were rapidly recognised to give generally improved growth of all types of plant cultures (Nitsch, 1969). EDTA has now become almost a standard medium component and is generally preferred to other alternative chelating agents (Table 3.8).

Preparation and use. (Fe)-EDTA chelates for tissue cultures are prepared in either of two ways.

- A ferric or ferrous salt is dissolved in water with EDTA and the solution is heated;
- A ready-prepared salt of iron salt of EDTA is dissolved and heated.

Heating can take place during the preparation of chelate stock solutions, or during the autoclaving of a medium.

The form of iron complexed is invariably Fe(III). If iron has been provided from ferrous salts, it is oxidised during heating in aerated solutions. The rate of oxidation of the ferrous ion is enhanced in some complexes and retarded in others (Albert, 1958). That of Fe^{2+}-EDTA is extremely rapid (Kolthoff and Auerbach (1952). Only a small proportion of Fe^{2+} is likely to remain: its chelate with EDTA is much less stable than the Fe(III) complex. Iron is however thought to be absorbed into plants in the ferrous form. Uptake of iron from EDTA probably occurs when molecules of Fe(III)-chelate bind to the outer plasma membrane (the plasmalemma) of the cytoplasm, where Fe(III) is reduced to Fe(II) and freed from the chelate (Chaney et al., 1972; Römheld and Marschner, 1983).

In most recent plant tissue culture work, EDTA has been added to media at an equimolar concentration with iron, where it will theoretically form a chelate with all the iron in solution. However, it has been found in practice that the Fe(III)-EDTA chelate, although stable at pH 2-3, is liable to lose some of its bound iron in culture media at higher pH levels; the displaced iron may form insoluble ferric hydroxides and iron phosphate (Dalton *et al.,* 1983). If this occurs, free EDTA will tend to form chelates with other metal ions in solution. Some micronutrients complexed with EDTA may then not be available to the plant tissues. Re-complexing may also happen if the EDTA to Fe ratio is increased by decreasing the amount of iron added to the medium (as has been proposed to solve the precipitation problem, see Chapter 4). It is not possible to add very much more than 0.1 mM EDTA to culture media because the chelating agent can become toxic to some plants (see below).

Hill-Cottingham and Lloyd-Jones (1961) showed that tomato plants absorbed iron from FeEDTA more rapidly than they absorbed EDTA itself, but concluded that both Fe and Fe-chelate were probably taken up. They postulated that EDTA liberated by the absorption of Fe, would chelate other metals in the nutrient solution in the order given at the beginning of Section 3.6.. Teasdale (1987) calculated that in many media, nearly all the copper and zinc, and some manganese ions might be secondarily chelated, but it is unclear whether micronutrients in this form are freely available to plant tissues. One presumes they are, for deficiency symptoms are not reported from *in vitro* cultures.

Ambiguous descriptions. In many early papers on plant tissue culture, the authors of scientific papers have failed to describe which form of EDTA was used in experiments, or have ascribed weights to EDTA, which should refer to its hydrated sodium salts. Singh and Krikorian (1980) drew attention to this lack of precision. They assumed that in papers where Na_2EDTA is described as a medium constituent, it indicates the use of the anhydrous salt (which would give 11 mol/l excess of EDTA to iron, with unknown consequences). However, the disodium salt of EDTA is generally made as the dihydrate (Beilstein's Handbuch der Organischen Chemie) and this is the form which will almost invariably have been used, Na_2EDTA merely being a shorthand way of indicating the hydrated salt without being intended as a precise chemical formula.

Further confusion has arisen through workers using ready-prepared iron-EDTA salts in media without specifying the weight or molar concentration of actual Fe used. Mono-, di-, tri-, and tetra-sodium salts of EDTA are possible, each with different (and sometimes alternative) hydrates, so that when a research report states only that a certain weight of 'FeEDTA' was used, it is impossible to calculate the concentration of iron that was employed with any certainty.

The compound 'monosodium ferric EDTA' with the formula NaFeEDTA (no water of hydration) exists, and is nowadays commonly selected as a source of chelated iron. However in some papers 'NaEDTA' has been used as an abbreviation for some other form of iron-EDTA salt. For example the paper of Eeuwens (1976) describing Y3 medium, says that to incorporate 0.05 mM iron, 32.5 mg/l 'sodium ferric EDTA' was used. The weight required using a compound with the strict molecular formula NaFeEDTA would be 18.35 mg/l. Hackett (1970) employed 'Na_4FeEDTA'. Gamborg and Shyluk (1981) and Gamborg (1982) said that to prepare B5 or MS medium with 0.1 mM Fe, 43 mg/l of 'ferric EDTA' or 'Fe-versenate' (EDTA) should be weighed. The compound recommended in these papers was probably the $Na_2FeEDTA.2H_2O$ chelate (theoretical mol. Wt. 428.2) as was the 'FeEDTA' (13% iron) employed by Davis *et al.,* (1977). It should be noted that NaFeEDTA is the only source of Na in MS medium apart from the contamination in the gelling agents.

Alternatives to EDTA. A few other chelating agents have been used in culture media in place of EDTA. The B5 medium of Gamborg *et al.,* (1968) was originally formulated with 28 mg/l of the iron chelate 'Sequestrene 330 Fe'. According to Heberle-Bors (1980), 'Sequestrene 330 Fe' is FeDTPA (Table 3.9), containing 10% iron (Anon, 1978). This means that the concentration of Fe in B5 medium was originally 0.05 mM. Gamborg and Shyluk (1981) have proposed more recently that the level of Fe should be increased to 0.1 mM. B5 medium is now often used with 0.1 mM FeEDTA, but some researchers still prefer FeDTPA, for example (Garton and Moses, 1986) used it in place of FeEDTA in Lloyd and McCown (1981) WPM medium for shoot culture of several woody plants.

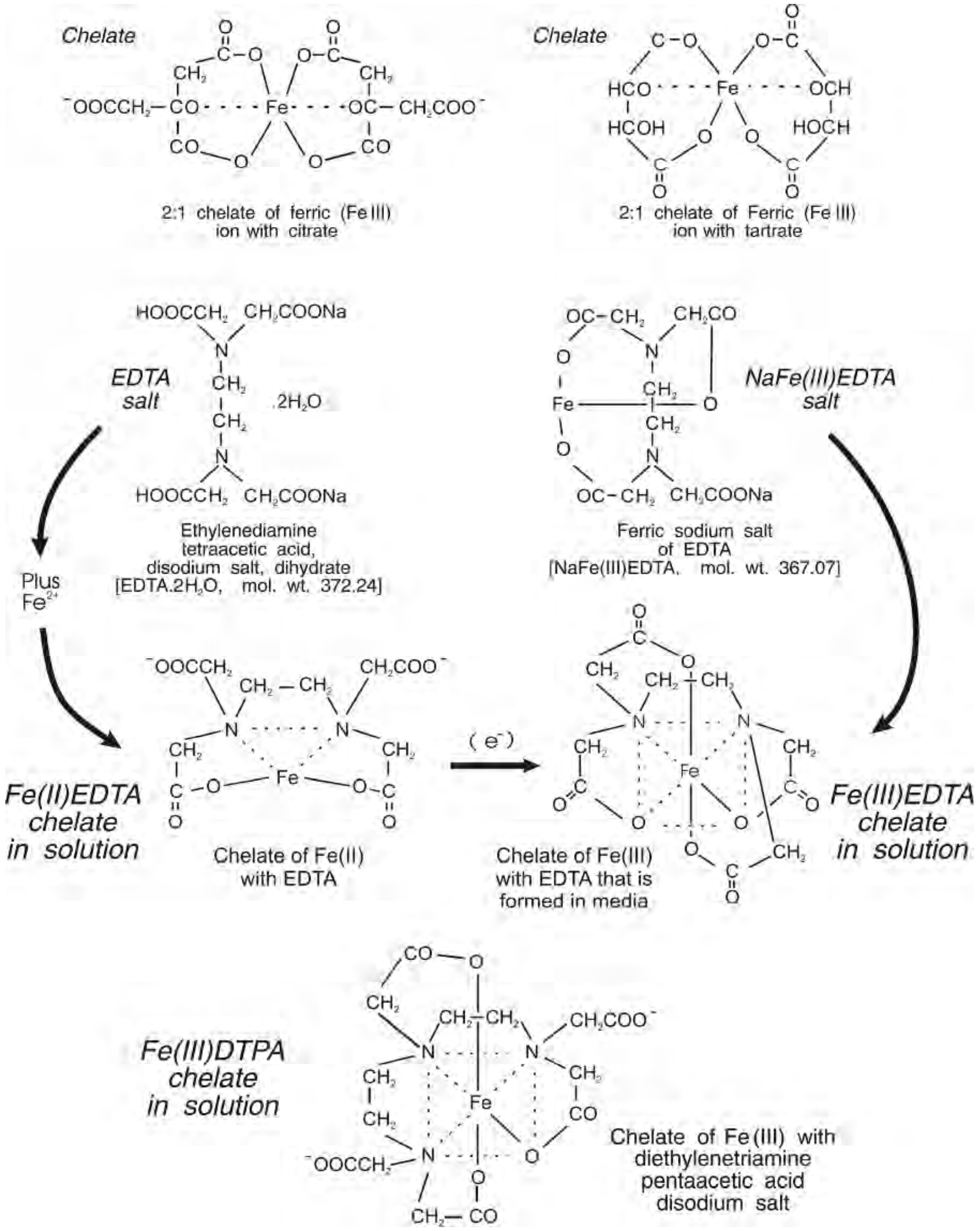

Fig. 3.6 Chemical structures of some chelating agents and iron chelates.

Growth regulatory effects of chelating agents. Although most iron, previously complexed to chelating agents such as EDTA, EDDHA and DTPA (Table 3.9) is absorbed as uncomplexed ions by plant roots, there is evidence that the chelating agents themselves can be taken up into plant tissues (Weinstein *et al.,* 1951; Tiffin *et al.,* 1960; Tiffin and Brown, 1961). Chelating compounds such as EDTA, in low concentrations, exert growth effects on plants, which are similar to those produced by auxins. The effects include elongation of oat coleoptiles (Heath and Clark, 1956a.b), and etiolated lupin hypocotyls (Weinstein *et al.,* 1956), the promotion of leaf epinasty (Weinstein *et al.,* 1956) and the inhibition of root growth (Burstrom, 1961, 1963). Hypotheses put

forward to explain these observations have included that:

- chelating agents act as auxin synergists by sequestering Ca from the cell wall (Thimann and Takahashi, 1958);
- the biological properties of the natural auxin IAA may be related to an ability to chelate ions; other chelating agents therefore mimic its action (Heath and Clark, 1960).

Burstrom (1960) noted that EDTA inhibited root growth in darkness (not in light) but that the growth inhibition could be overcome by addition of Fe^{3+} or several other metal ions (Burstrom, 1961). He recognised that reversal of EDTA action by a metal does not mean that the metal is physiologically active but that it might only release another cation, which had previously been made unavailable to the tissue by chelation.

Effects in tissue culture. Growth and morphogenesis in tissue cultures have been noted on several occasions to be influenced by chelating agents other than EDTA. It has not always been clear whether the observed effects were caused by the chelation of metal ions, or by the chelating agent *per se*.

The growth rate of potato shoot tips was increased by 0.01-0.3 mg/l 8-hydroxyquinoline (8-HQ) when cultured on a medium which also contained EDTA (Goodwin, 1966), and more callus cultures of a haploid tobacco variety formed shoots in the absence of growth regulators when DHPTA was added to Kasperbauer and Reinert (1967) medium which normally contains 22.4 mg/l EDTA. The DHPTA appears to have been used in addition to the EDTA, not as a replacement, and was not effective on callus of a diploid tobacco (Kochhar *et al.,* 1970). In the same experimental system, Fe-DHPTA and Fe-EDDHA were more effective in promoting shoot formation from the haploid-derived tissue than Fe with CDTA, citric acid or tartaric acid (Kochhar *et al.,* 1971).

The inclusion of EDTA into a liquid nutrient medium caused the small aquatic plant *Lemna perpusilla* to flower only in short day conditions whereas normally the plants were day-neutral (Hillman, 1959, 1961). In the related species *Wolffia microscopica*, plants did not flower unless EDTA was present in the medium, and then did so in response to short days (Maheshwari and Chauhan, 1963). When, however, Maheshwari and Seth (1966) substituted Fe-EDDHA for EDTA and ferric citrate, they found that plants not only flowered more freely under short days, but also did so under long days. The physiological effect of EDTA and EDDHA as chelating agents was thus clearly different. This was again shown by Chopra and Rashid (1969) who found that the moss *Anoectangium thomsonii* did not form buds as other mosses do, when grown on a simple medium containing ferric citrate or Fe-EDTA, but did so when 5-20 mg/l Fe-EDDHA was added to the medium instead. An optimum concentration was between 5 and 8 mg/l. Rashid also discovered that haploid embryoids developed more freely from *in vitro* cultures of *Atropa belladonna* pollen microspores when Fe-EDDHA was incorporated into the medium, rather than Fe-EDTA (Rashid and Street, 1973). Heberle-Bors (1980) did not obtain the same result, and found that FeEDTA was superior to FeEDDHA for the production of pollen plants from anthers of this species and of two Nicotianas. In tobacco, the production of haploid plants was greatest with FeEDTA, next best with FeDTPA, FeEGTA, FeEDDHA, and poorest with Fe citrate. Each complex was tested at or about the same iron concentration. Heberle-Bors also showed that chelating agents are differentially absorbed by activated charcoal (see Chapter 7). In tissue culture of rose (Van Der Salm, 1994), *Prunus* (Mallosiotis *et al.,* 2003), citrus (Dimassi *et al.,* 2003) and red raspberry (Zawadzka and Orlikowska, 2006), it is advantageous to use FeEDDHA rather than FeEDTA.

Toxicity caused by chelating agents. Although low concentrations of EDTA markedly stimulate the growth of whole plants in hydroponic cultures by making iron more readily available, the compound begins to be toxic at higher levels. By comparisons

Table 3.9 Some common chelating agents.

EDTA	Etyhylenediaminetetraaceticacid
EGTA	Ethyleneglycol-bis(2-aminoethylether)tetraaceticacid
EDDHA	Etyhylenediamine-di(*o*-hydroxyphenyl)acetic acid
DTPA	Dietyhylenetriaminepentaacetic acid
DHPT	1,3-diamino-2-hydroxypropane-tetraaceticacid

with observations on animal tissues, Weinstein *et al.,* (1951) suggested that toxicity arose through competition between EDTA and enzymes (and other physiologically-active complexes) in the plant, for metals essential to their activity. This will occur if the avidity of the chelating agent is greater than the metal binding capacity of proteins on the surface of cells (Albert, 1958).

Toxicity can also occur in *in vitro* cultures. Legrand (1975) found that an optimum rate of adventitious shoot initiation occurred in endive leaf segments when only 7.5 mg/l EDTA (one fifth the concentration used in MS medium) was employed. In these circumstances, higher levels of EDTA were clearly inhibitory and more than 55 mg/l prevented shoot formation. Dalton *et al.,* (1983) found that 0.3 mM EDTA (compared to the 0.1 mM in MS medium) reduced the growth rate of *Ocimum* cell suspensions.

Flower buds of *Begonia franconis* died within a few days if cultured with a high level of FeEDTA (1-1.5 mM, i.e. 10-15 times the normal level) together with 0.4-1.6 mM $H_2PO_4^-$. Berghoef and Bruinsma (1979a) thought that Fe^{3+} released from the FeEDTA complex, had precipitated the phosphate. Necrosis was avoided by increasing $H_2PO_4^-$ concentration to 6.4 mM.

Tissues may be damaged by culture in media containing synthetic chelating agents where the pH approaches neutrality, because at these pH levels, EDTA and EGTA have been shown to remove calcium ions from the membranes of mitochondria and this inhibits NAD(P)H oxidation and respiration (Moller and Palmer, 1981). Chelating agents have been found to inhibit the action of the growth substance ethylene (see Chapter 7) and are thought to do so by sequestering Cu ions within plant tissues, thereby interfering with the synthesis or action of a Cu-containing enzyme responsible for ethylene metabolism. EDTA can also inhibit the activity of plant polyphenol oxidase enzymes *in vitro* (Weinstein *et al.,* 1951) and Smith (1968) thought that this might occur because EDTA made Cu ions less available for enzyme incorporation, when he found the chelating agent was able to prevent the blackening of freshly-isolated *Carex flacca* shoot tips. Several oxidative reactions are also biochemically catalysed by ions such as Cu^{2+}, Co^{2+} and Zn^{2+}, and where this is the case [e.g. the oxidation of glutathione – Martin (1979); catechol amine oxidation – Kiss and Gergely (1979)], chelating agents such as EDTA and CDTA are inhibitory.

REFERENCES

ABOU-MANDOUR A. 1977 Pharmaceutical-biological studies of the genus *Harpagophytum*. 2. Communication: Tissue cultures of *Harpagophytum procumbens*. Planta Med. 31, 238-244.

ADATIA M.H. & BESFORD R.T. 1986 The effect of silicon on cucumber plants grown in recirculating nutrient solution. Ann. Bot. 58, 343-351.

ALBERT A. 1958 Metal-binding agents in chemotherapy: the activation of metals by chelation. pp. 112-138 in Cowan and Rowatt (eds.) *The Strategy of Chemotherapy*. Soc. for Gen. Microbiol. 8th Symp. Cambridge Univ. Press, Cambridge.

ALDERSON P.G., HARBOUR M.A. & PATIENCE P.A. 1987 Micropropagation of *Prunus tenella* cv. Firehill. Acta Hortic. 212, 463-468.

ALI A.H.N. & JARVIS B.C. 1988 Effects of auxin and boron on nucleic acid metabolism and cell division during adventitious root regeneration. New Phytol. 108, 383- 391.

AMIN M.N. & JAISWAL V.S. 1988 Micropropagation as an aid to rapid cloning of a guava cultivar. Scientia Hort. 36, 89-95.

AMMIRATO P.V. & STEWARD F.C. 1971 Some effects of the environment on the development of embryos from cultured free cells. Bot. Gaz. 132, 149-158.

ANDERSON J.O. 1976 Embryogenesis in wild carrot cells. In Vitro 12, 332.

ANON 1978 *Farm Chemicals Handbook*, 1978. Meister Publ.Co. Willoughby, Ohio 44094.

ANSTIS P.J.P. & NORTHCOTE D.H. 1973 The initiation, growth and characteristics of a tissue culture from potato tubers. J. Exp. Bot. 24, 425-441.

ARMSTRONG C.L. & GREEN C.E. 1985 Establishment and maintenance of friable embryogenic maize callus and involvement of L-proline. Planta 164, 207-214.

ARNON D.I. & STOUT P.R. 1939 Molybdenum as an essential element for higher plants. Plant Physiol. 14, 599-602.

ARNOZIS P.A., NELEMANS J.A. & FINDENEGG G.R. 1988 Phosphoendpyruvate carboxylase activity in plants grown with either NO_3^- or NH_4^+ as inorganic nitrogen source. J. Plant Physiol. 132, 23-27.

ASAHIRA T. & KANO Y. 1977 Shoot formation from cultured tissue of strawberry fruits. J. Jap. Soc. Hortic. Sci. 46, 317-324.

ASOKAN M.P., O'HAIR S.K. & LITZ R.E. 1983 *In vitro* plant development from bulbil explants of two *Dioscorea* species. HortScience 18, 702-703.

AVILA A.D., PEREYRA, S.M. & ARGÜELLO J.M. 1998 Nitrogen concentration and proportion of NH_4^+-N affect potato cultivar response in solid and liquid media. HortScience 33, 336-338.

BANNERJEE S. & GUPTA S. 1976 Embryogenesis and differenttiation in *Nigella sativa* leaf callus *in vitro*. Physiol. Plant. 38 115-120.

BARGHCHI M. 1986 *In vitro* micropropagation of *Pistacia* rootstocks. Comb. Proc. Int. Plant Prop. Soc. 1985 35, 334-337.

BARGHCHI M. & ALDERSON P.G. 1996 The control of shoot tip necrosis in *Pistacia vera* L. *in vitro*. Plant Growth Reg. 20, 31-35.

BARKER A.V. & COREY K.A. 1987 Ammonium-induced ethylene evolution by horticultural crops. HortScience 22, 381.

BARROSO M., LEVA A.R. & MURILLO J.M. 1985 La multiplicazione del melo con la tecnica della micropropagazione. Influenza del mezza nutritivo sul contenuto di alcumi elementi

minerali nel callo e negli espianti di mela cv. Golden Delicious. Riv. Ortoflorofrutti. Ital. 69, 123-131.

BARWALE U.B., KERNS H.R. & WIDHOLM J.M. 1986 Plant regeneration from callus cultures of several soybean genotypes via embryogenesis and organogenesis. Planta 167, 473-481.

BASU A., SETH U. & GUHA-MUKHERJEE S. 1989 Regulation of cell proliferation and morphogenesis by amino acids in *Brassica* tissue cultures and its correlation with threonine deaminase. Plant Cell Rep. 8, 333-335.

BAYLEY J.M., KING J. & GAMBORG O.L. 1972a The effect of the source of inorganic nitrogen on growth and enzymes of nitrogen assimilation in soybean and wheat cells in suspension cultures. Planta 105, 15- 24.

BAYLEY J.M., KING J. & GAMBORG O.L. 1972b The ability of amino compounds and conditioned medium to alleviate the reduced nitrogen. Planta 105, 25-32.

BEASLEY C.A., TING I.P., LINKINS A.E., BIRNBAUM E.H. & DELMER D.P. 1974 Cotton ovule culture: A review of progress and a preview of potential. pp. 169-192 in Street H.E. (ed.) *Tissue Culture and Plant Science.* Academic Press, London, New York, San Francisco.

BEHKI R.M. & LESLEY S.M. 1980 Shoot regeneration from leaf callus of *Lycopersicon esculentum*. Z. Pflanzenphysiol. 98, 83-87.

BEHREND J. & MATELES R.I. 1976 Nitrogen metabolism in plant cell suspension cultures. II. Role of organic acids during growth in ammonia. Plant Physiol. 58, 510-512.

BEHRENDT U. & ZOGLAUER K. 1996 Boron controls suspensor development in embryogenic cultures of *Larix decidua*. Physiol. Plant. 97, 321-326.

BEN-ZIONI A., VAADIA Y. & LIPS S.H. 1971 Nitrate uptake by roots as regulated by nitrate reduction products of the shoots. Physiol. Plant. 24, 288-290.

BERGHOEF J. & BRUINSMA J. 1979a Flower development of *Begonia franconis* Liebm. II. Effects of nutrition and growth-regulating substances on the growth of flower buds *in vitro*. Z. Pflanzenphysiol. 93, 345-357.

BERGHOEF J. & BRUINSMA J. 1979b Flower development of *Begonia franconis* Liebm. IV. Adventitious flower bud formation in excised inflorescence pedicels *in vitro*. Z. Pflanzenphysiol. 94, 407- 416.

BERTHELOT A. 1934 Nouvelles remarques d'ordre chimique sur le choix des milieux de culture maturels et sur la manière de formules des milieux synthétiques. Bull. Soc. Chim. Biol. Paris 16, 1553- 1557.

BERUTO M., CURIR P. & DEBERGH P 1999 Influence of agar on *in vitro* cultures II. Biological performance of *Ranunculus* on media solidified with three different agar brands. In Vitro Cell. Dev. Biol. Plant 35, 94-101.

BIEDERMANN I.E.G. 1987 Factors affecting establishment and development of *Magnolia* hybrids *in vitro*. Acta Hortic. 212, 625-629.

BIRNBAUM E.H., BEASLEY C.A. & DUGGER W.M. 1974 Boron deficiency in unfertilized cotton (*Gossypium hirsutum*) ovules grown *in vitro*. Plant Physiol. 54, 931-935.

BISTER-MIEL F., GUIGNARD J.-L., BURY M. & AGIER C. 1985 Glutamine as an active component of casein hydrolysate: its balancing effect on plant cells cultured in phosphorus deficient medium. Plant Cell Rep. 4, 161-163.

BHOJWANI S.S., MULLINS K. & COHEN D 1987. Micropropagation of *Feijoa sellowiana* Berg. Acta Hort 212, 69-76.

BLEVINS D.G. & LUKASZEWSKI K.M. 1998 Boron in plant structure and function. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 95-122.

BOBTELSKY M. & JORDAN J. 1945 The metallic complexes of tartrates and citrates, their structure and behavior in dilute solutions. I. The cupric and nickelous complexes. J. Am. Chem. Soc. 67, 1824-1831.

BONGA J.M. 1984 Adventitious shoot formation in cultures of immature female strobili of *Larix decidua*. Physiol. Plant. 62, 416-421.

BONNER J. & ADDICOTT F. 1937 Cultivation *in vitro* of excised pea roots. Bot. Gaz. 99, 144-170.

BOUHARMONT J. 1961 Embryo culture of rice on a sterile medium. Euphytica 10, 283-293.

BOULAY M. 1987 *In vitro* propagation of tree species. pp. 367-382 in Green C.E, Sommers D.A., Hackett W.P. and Biesboer D.D. (eds.) *Plant Tissue and Cell Culture*. Alan R. Liss, Inc., New York.

BOUMAN H. & TIEKSTRA A. 2005 Adaptations of the mineral composition of tissue culture media on the basis of plant elemental analysis and composition of hydroponic substrates. In: Hvoslef-Eide, A.K. and Preil W. (eds) *Liquid Culture Systems for in vitro Plant Propagation*. Springer, Dordrecht. Pp 493-505.

BOURGIN J.-P. & NITSCH J.P. 1967 Production of haploid *Nicotiana* from excised stamens. Ann. Physiol. Veg. 9, 377- 382.

BOV J.M., BOV C., WHATLEY F.R. & ARNON D.I. 1963 Chloride requirement for oxygen evolution in photosynthesis. Z. Naturforsch. Teil B 18, 683-688.

BOWEN J.E. 1979 Boron essentiality and transport in suspension cultured sugarcane cells. Plant Physiol. 63, Suppl., 163.

BOWEN M.R., HOWARTH J. & LONGMAN K.A. 1975 Effects of auxins and other factors on the rooting of *Pinus contorta* Dougl. cuttings. Ann. Bot. 39, 647-656.

BOXUS PH. 1974 The production of strawberry plants by *in vitro* micropropagation. J. Hortic. Sci. 49, 209-210.

BREWBAKER J.L. & KWACK B.H. 1963 The essential role of calcium ion in pollen germination and pollen tube growth. Am.J. Bot. 50, 859-865.

BRIDSON E.Y. 1978 Diets, culture media and food supplements. pp. 91-281 in Rechcigl M. Jr. (ed.) *CRC Handbook Series in Nutrition and Food*. Section G. Vol.3.

BROWN S., WITHERELL D.F. & DOUGALL D.K. 1976 The potassium requirement for growth and embryogenesis in wild carrot suspension cultures. Physiol. Plant. 37, 73-79.

BROWNELL P.F. 1979 Sodium as an essential micronutrient element for plants and its possible role in metabolism. Adv. Bot. Res. 7, 117-224.

BROYER T.C., CARLTON A.B., JOHNSON C.M. & STOUT P.R. 1954 Chlorine - a micronutrient element for higher plants. Plant Physiol. 29, 526-532.

BURKHOLDER P.R. & NICKELL L.G. 1949 Atypical growth of plants. I. Cultivation of virus tumors of *Rumex* on nutrient agar. Bot. Gaz. 110, 426-437.

BURSTROM H. 1960 Influence of iron and gibberellic acid on the light sensitivity of roots. Physiol. Plant. 13, 597-615.

BURSTROM H. 1961 Growth action of EDTA in light and darkness. Physiol. Plant. 14, 354-377.

BURSTROM H. 1963 Growth regulation by metals and chelates. Adv. Bot. Res. 1, 73-100.

BUWALDA F. & GREENWAY H. 1989 Nitrogen uptake and growth of wheat during O_2 deficiency in root media containing NO_3^- only, or NO_3^- plus NH_4^+. New Phytol. 111, 161-166.

CAMPBELL R.A. & DURZAN D.J. 1975 Induction of multiple buds and needles in tissue cultures of *Picea glauca*. Can. J. Bot. 53, 1652-1657.

CAPITANI F. & ALTAMURA M.M. 2004 Exogenous calcium enhances the formation of vegetative buds, flowers and roots in tobacco pith explants cultured in the absence of exogenous hormones. Plant Cell Tissue Organ Cult. 77,1-10.

CARMAN J.G., JEFFERSON N.E. & CAMPBELL W.F. 1988 Induction of embryogenic *Triticum aestivum* L. calli. I.

Quantification of genotype and culture medium effects. Plant Cell Tissue Organ Cult. 12, 83-95.

CATTOIR-REYNAERTS A., DEGRYSE E., NEGRUTIU I., AERTS M. & JACOBS M. 1981 Effects of aspartate-derived amino acids on growth of barley and *Arabidopsis* plants and callus. Z. Pflanzenphysiol. 101, 67-74.

CHAILLOU S. & CHAUSSAT R. 1986 Changes in mineral concentration of potato organ fragments. Phytomorph. 36, 263-270.

CHALEFF R.S. 1983 Induction, maintenance and differentiation of rice callus cultures on ammonium as sole nitrogen source. Plant Cell Tissue Organ Cult. 2, 29-37.

CHAND S. & ROY S.C. 1981 Induction of organogenesis in callus cultures of *Nigella sativa* L. Ann. Bot. 48, 1-4.

CHANEY R.L., BROWN J.C. & TIFFIN L.O. 1972 Obligatory reduction of ferric chelates in iron uptake by soybeans. Plant Physiol. 50, 208-213.

CHEE R. 1986 *In vitro* culture of *Vitis*: The effects of light spectrum, manganese sulfate and potassium iodide on morphogenesis. Plant Cell Tissue Organ Cult. 7, 121-134.

CHEN Z.1984 Chapter 19 ─ Rubber (*Hevea*). pp. 546-571 in Sharp W.R., Evans D.A., Ammirato P.V. and Yamada Y. (eds.) *Handbook of Plant Cell Culture*. Vol. 2. *Crop Species*. Macmillan Publishing Co., New York, London.

CHENG T.-Y. 1977 Factors effecting adventitious bud formation of cotyledon culture of douglas fir. Plant Sci. Lett. 9, 179-187.

CHENG T.-Y. 1978 Clonal propagation of woody species through tissue culture techniques. Comb. Proc. Int. Plant Prop. Soc. 28, 139-155.

CHIN C. & MILLER D. 1982 Some characteristics of the phosphate uptake by *Petunia* cells. HortScience 17, 488.

CHOPRA R.N. & RASHID A. 1969 Auxin-cytokinin interaction in shoot-bud formation of a moss, *Anoectangium thomsonii* Mitt. Z. Pflanzenphysiol. 61, 192-198.

CHROBOCZEK-KELKER H. & FILNER P. 1971 Regulation of nitrite reductase and its relationship to the regulation of nitrate reductase in cultured tobacco cells. Biochim. Biophys. Acta 252, 69-82.

CHU C.-C., WANG C.-C., SUN C.-S., HSU C., YIN K.-C., CHU C.-Y. & BI F.-Y. 1975 Establishment of an efficient medium for anther culture of rice, through comparative experiments on the nitrogen sources. Sci. Sinica 18, 659-668.

CHU C.C. & HILL R.D. 1988 An improved anther culture method for obtaining higher frequency of pollen embryoids in *Triticum aestivum* L. Plant Science 55, 175-181.

CIARROCCHI G., CELLA R. & NIELSEN E. 1981 Release of nucleotide- cleaving acid phosphatase from carrot cells grown in suspension culture. Physiol. Plant. 53, 375-377.

CLARKSON D.T. & HANSON J.B. 1980 The mineral nutrition of higher plants. Annu. Rev. Plant Physiol. 31, 239-298.

COOMBES A.J., PHIPPS N.W. & LEPP N.W. 1977 Uptake patterns of free and complexed copper in excised roots of barley (*Hordeum vulgare* L. var. Zephyr). Z. Pflanzenphysiol. 82, 435-439.

COREY K.A. & BARKER A.V. 1987 Physiology of ethylene evolution by tomato plants under ammmonium-induced stress. HortScience 22, 381.

CRAVEN G.A., MOTT R.L. & STEWARD F.C. 1972 Solute accumulation in plant cells. IV. Effects of ammonium ions on growth and solute content. Ann. Bot. 36, 897-914.

CREMIERE L., SBAY H. & PRAT D. 1987 *In vitro* culture of *Alnus* species. Acta Hortic. 212, 543-546.

CRUICKSHANK I.A.M., DUDMAN W.F., PEOPLES M.B. & SMITH M.M. 1987 Elicitation of pisatin in pea (*Pisum sativum* L.) by copper-asparagine complexes. Aust. J. Plant Physiol. 14, 549-559.

DAHLEEN, L.S. 1995 Improved plant regeneration from barley cultures by increased copper levels. Plant Cell Tissue Organ Cult. 43, 267-269.

DALTON C.C., IQBAL K. & TURNER D.A. 1983 Iron phosphate precipitation in Murashige and Skoog media. Physiol. Plant. 57, 472-476.

DAMIANO C. 1980 Strawberry micropropagation. pp. 11-22 in *Proceedings of the Conference on Nursery Production of Fruit Plants – Applications and Feasibility*. USDA, ARS, ARR-NE-11.

DAVID A. 1972 Effets de diverses solutions minérales sur la prolifération des tissus de Pin maritime en culture *in vitro*. Compt. Rend. Acad. Sci. Paris 275D, 2857-2860.

DAVIS D.G., OLSON P.A & STOLZENBERG R.L. 1988 Organogenesis in cell cultures of leafy spurge (Euphorbiaceae) accessions from Europe and North America. Plant Cell Rep. 7, 253-256.

DAVIS M.J., BAKER R. & HANAN J.J. 1977 Clonal multiplication of carnation by micropropagation. J. Am. Soc. Hortic. Sci. 102, 48-53.

DE GREEF W., DELON R., DE BLOCK M., LEEMANS J. & BOTTERMAN J. 1989 Evaluation of herbicide resistance in transgenic crops under field conditions. Nat. Biotechnol. 7, 61-64.

DE JONG A.W. & BRUINSMA J. 1974 Pistil development in *Cleome* flowers III. Effects of growth-regulating substances on flower buds of *Cleome iberidella* Welv. ex Oliv. grown *in vitro*. Z. Pflanzenphysiol. 73, 142-151.

DE JONG A.W., SMIT A.L. & BRUINSMA J. 1974 Pistil development in *Cleome* flowers II. Effects of nutrients on flower buds of *Cleome iberidella* Welv. ex Oliv. grown *in vitro*. Z. Pflanzenphysiol. 72, 227-236.

DE KLERK G.J. & WIJNHOVEN F. 2005 Water retention capacity of tissue cultured plants. Prop. Orn. Plants 5, 14-18.

DEBERGH P.C. 1988 Micropropagation of woody species -state of the art on *in vitro* aspects. Acta Hortic. 227, 287-295.

DENCSO I. 1987 Factors influencing vitrification of carnation and conifers. Acta Hortic. 212, 167-176.

DEVLIN R.M. 1975 *Plant Physiology*. 3rd. Edition. Van Nostrand Reinhold Co., New York.

DIMASSI K., CHOULIARAS V., DIAMANTIDIS G. & THERIOS I. 2003 Effect of iron and auxins on peroxidase activity and rooting performance of three citrus rootstocks *in vitro*. J. Plant Nutr. 26, 1023-1034.

DIXON N.E., GAZZOLA C., BLAKELEY R.L. & ZERNER B. 1975 Jack bean urease (EC 3.5.1.5). A metalloenzyme. A simple biological role for nickel? J. Am. Chem. Soc. 97, 4131-4133.

DOERSCHUG M.R. & MILLER C.O. 1967 Chemical control of adventitious organ formation in *Lactuca sativa* explants. Am. J. Bot. 54, 410-413.

DOUGALL D.K. & VERMA D.C. 1978 Growth and embryo formation in wild carrot suspension cultures with ammonium ion as a sole nitrogen source. In Vitro 14, 180-182.

DOUGALL D.K. & WEYRAUCH K.W. 1980 Abilities of organic acids to support growth and anthocyanin acumulation by suspension cultures of wild carrot cells, using ammonium as the sole nitrogen source. In Vitro 16, 969-975.

DOUGALL D.K. 1977 Current problems in the regulation of nitrogen metabolism in plant cell cultures. pp. 76-84 in Barz W., Reinhard E. and Zenk M.H. (eds.) *Plant Tissue Culture and its Biotechnological Application.* Springer-Verlag, Berlin, Heidelberg, New York.

DOUGALL D.K. 1981 Media factors affecting growth. pp. 277-280 in Constantin M.J., Henke R.R., Hughes K.W. and Conger B.V. *Propagation of Higher Plants through Tissue Culture: Emerging Technologies and Strategies*. Permagon Press, Oxford, New York, Sydney. ISBN 0098-8472.

DREW R.A. 1987 The effects of medium composition and cultural conditions on *in vitro* root initiation and growth of papaya (*Carica papaya* L.). J. Hortic. Sci. 62, 551-556.
DRIRA N. & BENBADIS A. 1975 Analyse, par culture d'anthères *in vitro*, des potentialités androgénétique de deux espèces de citrus (*Citrus medica* L. et *Citrus limon* L. Burm. Compt. Rend. Acad. Sci. Paris 281D, 1321-1324.
DRUART PH. 1988 Regulation of axillary branching in micropropagation of woody fruit species. Acta Hortic. 227, 369-380.
DURZAN D.J. 1976 Biochemical changes during gynosperm development. Acta Hortic. 56, 183-194.
DURZAN D.J. 1982 Nitrogen metabolism and vegetative propagation of forest trees. pp. 256-324 in Bonga J.M. and Durzan D.J (eds.) *Tissue Culture in Forestry*. Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, Boston, Lancaster. ISBN 90-247-2660-3.
DURZAN D.J. 1987 Ammonia: Its analogues, metabolic products and site of action in somatic embryogenesis. pp. 92-136 in Bonga J.M. and Durzan D.J (eds.) *Cell and Tissue Culture in Forestry*. Vol. 2. *Specific Principles and Methods: Growth and Develop-ment.* Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, Boston, Lancaster. ISBN 90-247-3431-2
EEUWENS C.J. 1976 Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera*) and cultured *in vitro*. Physiol. Plant. 36, 23-28.
EICHHORN G.L. 1980 The function of metal ions in genetic regulation. pp. 1-21 in Sigel H. (ed.) 1980 *Metal Ions in Biological Systems* 10. Marcel Dekker Inc. New York, Basel.
EINSET J.W. 1978 Citrus tissue culture - stimulation of fruit explant cultures with orange juice. Plant Physiol. 62, 885-888.
EL BADAOUI H., MORARD P. & HENRY M 1996 Stimulation of the growth and solamargine production by *Solanum paludosum* multiple shoot cultures using a new culture medium. Plant Cell Tissue Organ Cult. 45, 153-158.
ELTINGE E.T. & REED H.S. 1940 The effect of zinc deficiency upon the root of *Lycopersicon esculentum*. Am. J. Bot. 27, 331-335.
EPSTEIN E. 1971 *Mineral Nutrition of Plants. Principles and Perspectives*. John Wiley and Sons Inc., New York, London, Sydney, Toronto.
ERIKSSON T. 1965 Studies on the growth requirements and growth measurements of cell cultures of *Happlopapus gracilis*. Physiol. Plant. 18, 976-993.
ERNER Y. & REUVENI O. 1981 Promotion of citrus tissue culture by citric acid. Plant Physiol. 67, Suppl.), 27.
ESKEW D.L., WELCH R.M. & CARY E.E. 1983 Nickel: an essential micronutrient for legumes and possibly all higher plants. Science 222, 621-623.
EVANS D.A., SHARP W.R. & PADDOCK E.F. 1976 Variation in callus proliferation and root morphogenesis in leaf tissue cultures of *Glycine max* strain T 219. Phytomorph. 26, 379-384.
FAYE M., DAVID A. & LAMANT A. 1986 Nitrate reductase activity and nitrate accumulation in *in vitro* produced axillary shoots, plantlets and seedlings of *Pinus pinaster*. Plant Cell Rep. 5, 368-371.
FERGUSON I.B. & DRBAK B.K. 1988 Calcium, mineral nutrition and salinity. HortScience 23, 262-269.
FILNER P. 1965 Semi-conservative replication of DNA in a higher plant cell. Exp. Cell Res. 39, 33-39.
FINER J.J. & NAGASAWA A. 1988 Development of an embryo-genic suspension culture of soybean (*Glycine* max Merrill.). Plant Cell Tissue Organ Cult. 15, 125-136.
FLETCHER J.S. 1980 Influence of nitrate and sucrose supply on the growth and senescence of Paul's Scarlet rose cells. Plant Physiol. 65, Suppl., 37.
FLINN B. & WEBB D.T. 1986 Effects of media components and the timing of cytokinin application on caulogenesis from *Pinus strobus* embryonic explants. p. 36 in Abstracts VI Intl. Cong. Plant Tissue & Cell Culture. Minneapolis, Minn.
FREYTAG A.H., ANAND S.C., RAO-ARELL A.P. & OWENS L.D. 1988 An improved medium for adventitious shoot formation and callus induction in *Beta vulgaris* L. *in vitro*. Plant Cell Rep. 7, 30-34.
FRIES L. 1962 Vitamin B12 in *Pisum sativum* (L.). Physiol. Plant. 15, 566-571.
FUJIWARA A. (ed.) 1982 *Plant Tissue Culture* 1982. Proc. 5th. Int. Cong. Plant Tiss. Cell Cult., Japan. Assoc. Plant Tissue Culture, Japan.
FUKUNAGA Y., KING J. & CHILD J.J. 1978 The differential effects to TCA-cycle acids on the growth of plant cells cultured in liquid media containing various nitrogen sources. Planta 139, 199-202.
FURUHASHI K.F. & TAKAHASHI Y. 1982 Glutamate dehydrogenase of green tobacco callus tissue. pp. 241-242 in Fujiwara A. (ed.) 1982 (q.v.).
GALSTON A.W. & HILLMAN W.S. 1961 The degradation of auxin. *Encyclopedia of Plant Physiology* 14, 647-670.
GAMBORG O.L. & EVELEIGH D.E. 1968 Culture methods and detection of glucanases in suspension cultures of wheat and barley. Can. J. Biochem. 46, 417-421.
GAMBORG O.L. & SHYLUK J.P. 1970 The culture of plant cells with ammonium salts as a sole nitrogen source. Plant Physiol. 45, 598-600.
GAMBORG O.L. & SHYLUK J.P. 1981 Nutrition, media and characteristics of plant cell and tissue cultures. pp. 21-44 in Thorpe T.A. (ed.) *Plant Tissue Culture: Methods and Applications in Agriculture.* Academic Press, New York, London, Toronto, Sydney.
GAMBORG O.L. 1970 The effects of amino acids and ammonium on the growth of plant cells in suspension culture. Plant Physiol. 45, 372-375.
GAMBORG O.L. 1982 Callus and cell culture. pp. 1-9 in Wetter L.R. and Constabel F. (eds.) *Plant Tissue Culture Methods* (2^{nd} Ed.) National Res. Council of Canada, Prairie Regional Res. Lab., Saskatoon.
GAMBORG O.L. 1991 Media preparation. pp. 1-24 in *Plant Tissue Culture Manual A1* Kluwer Acad. Publ., The Netherlands.
GAMBORG O.L., CONSTABEL F. & SHYLUK J.P. 1974 Organogenesis in callus from shoot apices of *Pisum sativum*. Physiol. Plant. 30, 125-128.
GAMBORG O.L., MILLER R.A. & OJIMA K. 1968 Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151-158.
GARTON S. & MOSES M.S. 1986 Production of native plants in tissue culture. Comb. Proc. Int. Plant Prop. Soc. 1985 35, 306-315.
GAUTHERET R.J. 1939 Sur la possibilité de réaliser la culture indéfinie des tissus tubercule de carotte. Compt. Rend. Acad. Sci. Paris 208, 118.
GAUTHERET R.J. 1942 *Manuel Technique de Culture des Tissue végétaux*. Masson et Cie, Paris.
GEORGE E.F., PUTTOCK D.J.M. & GEORGE H.J. 1987 *Plant Culture Media Vol.1*. Exegetics Ltd., Westbury, England.
GEORGE E.F., PUTTOCK D.J.M. & GEORGE H.J. 1988 *Plant Culture Media Vol.2*. Exegetics Ltd., Westbury, England.
GERENDÁS J., POLACCO J.C., FREYEREMUTH S.K. & SATTELMACHER B. 1999 Signifcance of nickel for plant growth and metabolism. J. Plant Nutr. Soil Sc. 162, 241-256.
GERTSSON U.E. 1988 Influence of macronutrient composition, TIBA and dark treatment on shoot formation and nitrogen

content in petiole explants of *Senecio hybridus*. J. Hortic. Sci. 63, 497-502.

GLASSTONE V.F.C. 1947 Inorganic micronutrients in tomato root tissue culture. Am. J. Bot. 34, 218-224.

GOLDBACH H. & AMBERGER A. 1986 Influence of boron deficiency on ^{3}H-indole-3yl-acetic acid uptake and efflux in cell cultures of *Daucus carota* L. Plant Growth Reg. 4, 81-86.

GONÇALVES S., CORREIA P.J., MARTINS-LOUÇÃO M.A. & ROMANO A. 2005 A new medium formulation for *in vitro* rooting of carob tree based on leaf macronutrients concentration. Biol. Plant. 49, 277-280.

GONG M., VAN DE LUIT A.H., KNIGHT M.R. & TREWAVAS A.J. 1998 Heat-shock-induced changes in intracellular Ca^{2+} level in tobacco seedlings in relation to thermotolerance. Plant Physiol. 116, 429-437.

GOODWIN P.B. 1966 An improved medium for the rapid growth of isolated potato buds. J. Exp. Bot. 17, 590-595.

GRAY D.J., CONGER B.V. & HANNING G.E. 1984 Somatic embryogenesis in suspension and suspension-derived callus cultures of *Dactylis glomerata*. Protoplasma 122, 196-202.

GREEN C.E. & PHILLIPS R.L. 1975 Plant regeneration from tissue cultures of maize. Crop Sci. 15, 417-427.

GREENWOOD N.N. 1973 The Chemistry of Boron. *Pergamon Texts Inorganic Chemistry* Vol. 8. Pergamon Press, Oxford, New York, Toronto.

GRIMES H.D. & HODGES T.K. 1990 The inorganic NO_3^-–NH_4^+ ratio influences plant regeneration and auxin sensitivity in primary callus derived from immature embryos of Indica rice (*Oryza sativa* L.). J. Plant Physiol. 136, 362-367.

GRUSAK M.A. 2001 Plant macro- and micronutrient minerals. *Encyclopedia of life sciences*. Nature Publishing Group. www.els.net

GUAN H. & DE KLERK G.J. 2000 Stem segments of apple microcuttings take up auxin predominantly *via* the cut surface and not *via* the epidermis. Sci. Hortic. 86, 23-32.

GUPTA S., SEN B. & BHATTACHARYA S. 1987 Embryogenesis and differentiation in two species of *Trigonella*. Phytomorph. 37, 95-101.

HACKETT W.P. 1970 The influence of auxin, catechol and methanolic tissue extracts on root initiation in aseptically cultured shoot apices of the juvenile and adult forms of *Hedera helix*. J. Am. Soc. Hort. Sci. 95, 398-402.

HAGA K.I. & SODEK L. 1987 Utilization of nitrogen sources by immature soybean cotyledons in culture. Ann. Bot. 59, 597-601.

HAGEN C.E. & HOPKINS H.T. 1955 Ionic species in orthophosphate absorption by barley roots. Plant Physiol. 30, 193-199.

HAHLBROCK K. 1974 Correlation between nitrate uptake, growth and changes in metabolic activities of cultured plant cells. p. 363 in Street H.E. (ed.) *Tissue Culture and Plant Science.* Academic Press, London, New York, San Francisco.

HALPERIN W. & WETHERELL D.F. 1965 Ammonium requirement for embryogenesis *in vitro*. Nature 205, 519-520.

HALPERIN W. 1967 Population density effects on embryogenesis in carrot cell cultures. Exp. Cell Res. 48, 170-173.

HANNAY J.W. & STREET H.E. 1954 Studies on the growth of excised roots. III. The molybdenum and manganese requirement of excised tomato roots. New Phytol. 53, 68-80.

HANNAY J.W. 1956 A study of the micronutrient requirements of excised roots. Ph.D. Thesis, Univ. Manchester [from Street H.E. 1966 The nutrition and metabolism of plant tissue and organ cultures. pp. 533-629 in Willmer E.N. *Cells and Tissues in Culture – Methods and Physiology*. Vol 3. Academic Press, London, New York].

HARRIS G.P. 1956 Amino acids as sources of nitrogen for the growth of isolated oat embryos. New Phytol. 55, 253-268.

HEATH O.V.S. & CLARK J.E. 1956a Chelating agents as plant growth substances. A possible clue to the mode of action of auxin. Nature 177, 1118-1121.

HEATH O.V.S. & CLARK J.E. 1956b Chelating agents as growth substances. Nature 178, 600-601.

HEATH O.V.S. & CLARK J.E. 1960 Chelation in auxin action. J. Exp. Bot. 11, 167-187.

HEBERLE-BORS E. 1980 Interaction of activated charcoal and iron chelates in anther cultures of *Nicotiana* and *Atropa belladonna*. Z. Pflanzenphysiol. 99, 339-347.

HEIMER Y.M. & FILNER P. 1971 Regulation of the nitrate assimilation pathway in cultured tobacco cells. Biochim. Biophys. Acta 230, 362-372.

HELLER R. 1953 Researches on the mineral nutrition of plant tissues. Ann. Sci. Nat. Bot. Biol. Vg., 11th Ser. 14, 1-223.

HELLER R. 1955 Les besoins mineraux des tissus en culture. pp. 1-21 in *La Physiologie des Cultures de Tissus végétaux.* Union Internationale des Sciences Biol. Series B (Colloques) 20 (1954).

HEMBERG T. 1951 Rooting experiments with hypocotyls of *Phaseolus vulgaris* L. Physiol. Plant. 4, 358-369.

HEPLER P.K. & WAYNE R.O. 1985 Calcium and plant development. Annu. Rev. Plant Physiol. 36, 397-439.

HEW C.S., TING S.K. & CHIA T.F. 1988 Substrate utilization by *Dendrobium* tissues. Bot. Gaz. 149, 153-157.

HEWITT E.J. & SMITH T.A. 1975 *Plant Mineral Nutrition*. English University Press.

HEWITT E.J. 1948 Relation of manganese and some other metals to the iron status of plants. Nature 161, 489.

HIATT A.J. 1978 Critique of absorbtion and utilization of ammonium nitrogen by plants. pp. 191-199 in *Nitrogen in the Environment* Vol.2. *Soil-Plant-Nitrogen Relationships*. Academic Press, New York, San Francisco, London. ISBN 0-12-518402-6 (v.2).

HILDEBRANDT A.C., RIKER A.J. & DUGGAR B.M. 1946 The influence of the composition of the medium on growth *in vitro* of excised tobacco and sunflower tissue cultures. Am. J. Bot. 33, 591-597.

HILL-COTTINGHAM D.G. & LLOYD-JONES C.P. 1961 Absorption and breakdown of iron-ethylenediamine tetraacetic acid by tomato plants. Nature 189, 312.

HILLMAN W.S. 1959 Experimental control of flowering in *Lemna*. I. General methods. Photoperiodism in *L. pepusilla* 6746. Am. J. Bot. 46, 466-473.

HILLMAN W.S. 1961 Experimental control of flowering in Lemna. III. A relationship between medium composition and the opposite photoperiodic responses of *L. perpusilla* 6746 and *L. gibba* G3. Am. J. Bot. 48, 413-419.

HISAJIMA S. 1982 Multiple shoot formation from almond seeds and an excised single shoot. Agric. Biol. Chem. 46, 1091-1093.

HOAGLAND D.R. & SNYDER W.C. 1933 Nutrition of strawberry plant under controlled conditions. Proc. Am. Soc. Hortic. Sci. 30, 288-296.

HOLM-HANSEN O., GERLOFF G.C. & SKOOG F. 1954 Cobalt as an essential element for blue-green algae. Physiol. Plant. 7, 665-675.

HOSSAIN B., HIRATA N., NAGATOMO Y., AKASHI R. & TAKAKI H. 1997 Internal zinc accumulation is correlated with increased growth in rice suspension culture. J. Plant Growth Regul. 16, 239-243.

HUNAULT G. 1985 Organic acids, pH, ammonium and nitrate interactions on the growth of *Asparagus* tissues cultivated *in vitro*. Ann. Sci. Nat. Bot. Biol. Veg.13, 63-75.

INOUE M. & MAEDA E. 1982 Control of organ formation in rice callus using two-step culture method. pp. 183-184 in Fujiwara A. (ed.) 1982 (q.v.).

ISHII K. 1986 *In vitro* plantlet formation from adventitious buds on juvenile seedlings of Hinoki cypress (*Chamaecyparis obtusa*). Plant Cell Tissue Organ Cult. 7, 247-255.

JAKOBEK J.L., BACKHAUS R.A. & HERMAN K. 1986 Micropropagation of candellila, *Euphorbia antisyphilitica* Zucc. Plant Cell Tiss. Organ Cult. 7, 145-148.

JACOBSEN L., HANNAPEL R.J. & MOORE D.P. 1958 Non-metabolic uptake of ions by barley roots. Plant Physiol. 33, 278-282.

JACOBSON L. 1951 Maintenance of iron supply in nutrient solutions by a single addition of ferric potassium ethylene-diamine tetra-acetate. Plant Physiol. 26, 411-413.

JACOBSON L., COOPER B.R. & VOLZ M.G. 1971 The interaction of pH and aeration in Cl uptake by barley roots. Physiol. Plant. 25, 432-435.

JAMES D.J., PASSEY A.J. & MALHOTRA S.B. 1984 Organogenesis in callus derived from stem and leaf tissues of apple and cherry rootstocks. Plant Cell Tissue Organ Cult. 3, 333-341.

JANSEN M.A.K., BOOIJ H., SCHEL J.H.N. & DE VRIES S.C. 1990 Calcium increases the yield of somatic embryos in carrot embryogenic suspension cultures. Plant Cell Rep. 9, 221-223.

JARVIS B.C. 1986 Endogenous control of adventitious rooting in non-woody cuttings. pp. 191-222 in Jackson M.B. (ed.) *New Root Formation in Plants and Cuttings*. Martinus Nijhoff Publ. ISBN 90 247 3260 3.

JARVIS S.C. 1984 The effects of nitrogen supply on the absorption and distribution of copper in Red clover (*Trifolium pratense* L.) grown in flowing solution with a low, maintained concentration of copper. Ann. Bot. 53, 153-161.

JENSEN C.J. 1974 Produection of monoploids in barley: A progress report. pp. 153-190 in Kasha. K.J. (ed.) *Haploids in Higher Plants: Advances and Potential.* Univ. Guelph, Canada.

JOHNSON C.M., STOUT P.R., BROYER T.C. & CARLTON A.B. 1957 Comparative chlorine requirements of different plant species. Plant Soil 8, 337-353.

JONES J.B. & MURASHIGE T. 1974 Tissue culture propagation *Aechmea fasciata* Baker and other bromeliads. Comb. Proc. Int. Plant Prop. Soc. 24, 117-126.

JORDAN D.B. & FLETCHER J.S. 1979 The relationship between NO_2^- accumulation, nitrate reductase and nitrite reductase in suspension cultures of Paul's Scarlet rose. Plant Sci. Lett. 17, 95-99.

JOSTEN P. & KUTSCHERA U. 1999 The micronutrient boron causes the development of adventitous roots in sunflower cuttings. Ann. Bot. 84, 337-342.

KAMADA H. & HARADA H. 1977 Influence of several growth regulators and amino acids on *in vitro* organogenesis of *Torenia* and *Daucus*. Acta Hortic. 78, 175-176.

KAMADA H. & HARADA H. 1979 Studies on the organogenesis in carrot tissue cultures. II. Effects of amino acids and inorganic nitrogenous compounds on somatic embryogenesis. Z. Pflanzen-physiol. 91, 453-463.

KAMADA H. & HARADA H. 1982 Studies on nitrogen metabolism during somatic embryogenesis in carrot. pp. 115-116 in Fujiwara A.(ed.) 1982 (q.v.).

KAMO K.K., BECWAR M.R. & HODGES T.K. 1985 Regeneration of *Zea mays* L. from embryogenic callus. Bot. Gaz. 146, 327-334.

KAO K.N. & MICHAYLUK M.R. 1975 Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126, 105-110.

KASPERBAUER M.J. & REINERT R.A. 1967 Photometrically assayable phytochrome *in vivo* in callus tissue cultured from *Nicotiana tabacum*. Physiol. Plant. 20, 977-981.

KING P.J. 1977 Studies on the growth in culture of plant cells, growth limitiation by nitrate and glucose in a chemostat culture of *Acer pseudoplatanus*. J. Exp. Bot. 28, 142-155.

KINTZIOS S., DROSSOPOULOS J.B. & LYMPEROPOULOS C. 2001 Effect of vitamins and inorganic micronutrients on callus growth and somatic embryogenesis from leaves of chilli pepper. Plant Cell Tissue Organ Cult. 67, 55-62.

KINTZIOS S., STAVROPOPOULOU ER. & SKAMNELI S. 2004 Accumulation of selected macronutrients and carbohydrates in melon tissue cultures: association with pathways of *in vitro* dedifferentiation and differentiation (organogenesis, somatic embryogenesis). Plant Sci. 167, 655-664.

KIRBY E.G. 1982 The effects of organic nitrogen sources on growth of cell cultures of Douglas fir. Physiol. Plant. 56, 114-117.

KIRBY E.G., LEUSTEK T. & LEE M.S. 1987 Nitrogen nutrition. pp. 67-88 in Bonga and Durzan (eds.) *Cell and Tissue Culture in Forestry* Vol 1. *General Principles and Biotech-nology*. Martinus Nijhoff Publishers, Dordrecht, Boston, Lancaster. ISBN 90-247-3430-4.

KISS T & GERGELY A. 1979 Complexes of 3,4-dihydroxyphenyl derivatives III. Equilibrium study of parent and some mixed ligand complexes of dopamine, alanaine and pyrocatechol with nickel(II), copper(II) and zinc(II) ions. Inorg. Chim. Acta, **36**, 31

KLAPHECK S., GROSSE W. & BERGMANN L. 1982 Effect of sulphur deficiency on protein synthesis and amino acid accumulation in cell suspension cultures of *Nicotiana tabacum*. Z. Pflanzenphysiol. 108, 235-245.

KLEIN R.M. & MANOS G.E. 1960 Use of metal chelates for plant tissue cultures. Ann. New York Acad. Sci. 88, 416-425.

KLEIN R.M., CAPUTO E.M. & WITTERHOLT B.A. 1962 The role of zinc in the growth of plant tissue cultures. Am. J. Bot. 49, 323-327.

KNIGHT M.R., CAMPBELL A.K., SMITH T.M. & TREWAVAS A.J. 1991 Transgenic plant aequorin reports the effects of touch, cold-shock and elicitors on cytoplasmic calcium. Nature 352, 424-426.

KNUDSON L. 1922 Non-symbiotic germination of orchid seeds. Bot. Gaz. 73, 1-25.

KNUDSON L. 1943 Nutrient solutions for orchid seed germin-ation. Am. Orchid Soc. Bull. 12, 77-79.

KOCHHAR T., BHALLA P. & SABHARWAL P. 1970 Formation de bourgeons végétatifs par des cals de tabac sous l'influence d'un agent de chélation: l'acide 1,3-diamino-2-hydroxypropane-N,N,N,N,-tetracétique (DNPTA). Compt. Rend. Acad. Sci. Paris 271D, 1619-1622.

KOCHHAR T.S., BHALLA P.R. & SABHARWAL P.S. 1971 Induction of vegetative buds in tobacco callus by chelating agents. Am. J. Bot. 58, 453.

KOETJE D.S., GRIMES H.D., WANG Y.-C. & HODGES T.K. 1989 Regeneration of indica rice (*Oryza sativa* L.) from primary callus from immature embryos. J. Plant Physiol. 13, 184-190.

KOLTHOFF I.M. & AUERBACH C. 1952 Studies on the system iron-ethylenediamine tetraacetate. J. Am. Chem. Soc. 74, 1452-1456.

KROSING M. 1978 Tissue culture under conditions of boron deficiency. Z. Pflanz. Bodenkunde 141, 523-534.

LAKSHMANA RAO R.V. & DE D.N. 1987 Haploid plants from *in vitro* anther culture of the leguminous tree, *Peltophorum pterocarpum* (DC) K. Hayne (Copper pod). Plant Cell Tiss. Organ Culture 11, 167-177.

LAMOTTE C.E. & LERSTEN N.R. 1971 An attempt to induce bacteria-free plants of *Psychotria punctata* (Rubiaceae) in tissue culture. Am. J. Bot. 58, 476.

LARKIN P.J. 1982 Sugarcane tissue and protoplast culture. Plant Cell Tissue Organ Cult. 1, 149-164.

LAVEE S. & HOFFMAN M. 1971 The effect of potassium ions on peroxidase activity and its isoenzyme composition as related to apple callus growth *in vitro*. Bot. Gaz. 132, 232-237.

LAZAR M.D. & COLLINS G.B. 1981 Comparative glutamine metabolism in tobacco callus and leaf tissues. Environ. Exp. Bot. 21, 425.

LEA P.J., HUGHES J.S. & MIFLIN B.J. 1979 Glutamine- and asparagine-dependent protein synthesis in maturing legume cotyledons cultured *in vitro*. J. Exp. Bot. 30, 529-537.

LEGRAND B. 1975 Action of iron and EDTA on the neoformation of buds, by the leaf fragments of endive cultivated *in vitro*. Compt. Rend. Acad. Sci. Paris 280D, 2215-2218.

LEIFFERT C., MURPHY K.P. & LUMSDEN P.J. 1995 Mineral and carbohydrate nutrition of plant cell and tissue cultures. Crit. Rev. Plant Sci. 14, 83-109

LENEE P. & CHUPEAU Y. 1989 Development of nitrogen assimilating enzymes during growth of cells derived from protoplasts of sunflower and tobacco. Plant Sci. 59, 109-117.

LERCH K. 1981 Copper monooxygenases: tyrosine and dopamine b-monooxygenase. pp. 143-186 in Sigel H. (ed.) 1981 Metal Ions in Biological Systems 13 Marcel Dekker Inc. New York, Basel.

LEWIS D.H. 1980 Boron, lignification and the origin of vascular plants - a unified hypothesis. New Phytol. 84, 209-229.

LI C., PFEFFER H., DANNEL F., RÖMHELD V. & BANGHERTH F. 2001 Effects of boron starvation on boron compartmentation, and possibly hormone-mediated elongation growth and apical dominance of pea (*Pisum sativum*) plants. Physiol. Plant. 111, 212-219.

LINSMAIER E.M. & SKOOG F. 1965 Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18, 100-127.

LITVAY J.D., JOHNSON M.A., VERMA D., EINSPAHR D. & WEYRAUCH K. 1981 Conifer suspension culture medium development using analytical data from developing needs. IPC Technical paper series, no.115.

LLOYD G. & McCOWN B. 1981 Commercially-feasible micropropagation of Mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Int. Plant Prop. Soc. Proc. 30, 421-427.

LOPEZ-BUCIO J., CRUZ-RAMIREZ A. & HERRERA-ESTRELLA L. 2003 The role of nutrient availability in regulating root architecture. Curr. Opin. Plant Biol. 6, 280-287.

LORETI F., MORINI S. & CONCETTI S. 1988 Effect of potassium and nitrogen concentration on growth of peach shoots cultured *in vitro*. Acta Hortic. 227, 311-317.

LUCIANI G.F., MARINANGELI P.A. & CURVETTO N.R. 2001 Increasing nitrate/ammonium ratio for improvement of garlic micropropagation. Sci. Hortic. 87, 11-20.

LUMSDEN P.J., PRYCE S. & LEIFERT C. 1990 Effect of mineral nutrition on the growth and multiplication of *in vitro* cultured plants. pp. 108-113 in Nijkamp H.I.J., Van Der Plas I.H.W. and Van Aartrijk J. (eds.) *Progress in Plant Cellular and Molecular Biology*. Proc. VIIth Int. Cong. on Plant Tissue and Cell Culture. Amsterdam, The Netherlands. 24-29 June 1990. Kluwer Academic Publishers, Dortrecht, Netherlands.

MACCARTHY J.J., RATCLIFFE D. & STREET H.E. 1980 The effect of nutrient composition on the growth cycle of *Catharanthus roseus* G. Don cells grown in batch culture. J. Exp. Bot. 31, 1315-1325.

MACKLON A.E.S. & SIM A. 1976 Cortical cell fluxes and transport to the stele in excised root segments of *Allium cepa* L. III. Magnesium. Planta 128, 5-9.

MACLACHLAN G.A. & WAYGOOD E.R. 1956 Catalysis of indoleacetic acid oxidation by manganic ions. Physiol. Plant. 9, 321-330.

MAHESHWARI S.C & CHAUHAN O.S. 1963 *In vitro* control of flowering in *Wolffia microscopica*. Nature 198, 99-100.

MAHESHWARI S.C. & SETH P.N. 1966 Induction of flowering in *Wolffia microscopica* by the iron salt of ethylene-di-*o*-hydroxyphenylacetic acid (Fe-EDDHA). Z. Pflanzenphysiol. 55, 89-91.

MALLOSIOTIS A.N., DIMASSI K., THERIOS I. & DIAMANTIDIS G. 2003 Fe-EDDHA promotes rooting of rootstoock GF-677 (*Prunus amygdalus* x *P. persica*) explants *in vitro*. Biol. Plant. 47, 141-144.

MARGARA J. & LEYDECKER M.-T. 1978 Différents types d'organogenèse observés chez le Colza, *Brassica napus* L. var. *oleifera* Metzg. Compt. Rend. Acad. Sci. Paris 287D, 17-20.

MARGARA J. & RANCILLAC M. 1966 Observations préliminaires sur le rôle du milieu nutritif dans l'initiation florale des bourgeons néoformés *in vitro* chez *Cichorium intybus* L. Compt. Rend. Acad. Sci. Paris 263D, 1455-1458.

MARGARA J. 1969a Étude des facteurs de la néoformation de bourgeons en culture *in vitro* chez le chou-fleur (*Brassica oleracea* L. var. *botrytis*). Ann. Physiol. Veg. 11, 95-112.

MARGARA J. 1969b Étude préliminaire des facteurs de la néoformations de bourgeons chez le chou-fleur (*Brassica oleracea* L. var. *botrytis*). Compt. Rend. Acad. Sci. Paris 268D, 686-688.

MARTIN R.B. 1979 Complexes of a-amino acids with chelatable side chain donor atoms. pp. 1-39 in Sigel H. (ed.) 1979 *Metal Ions in Biological Systems 9* Marcel Dekker Inc., New York and Basel.

MARTIN S.M., ROSE D. & HUI V. 1977 Growth of plant cell suspensions with ammonium as the sole nitrogen source. Can. J. Bot. 55, 2838-2843.

MARTINELLI A. 1988 Use of *in vitro* techniques for selection and cloning of different *Pistacia* species. Acta Hortic. 227, 436-437.

MASHAYEKHI K. & NEUMANNN K.H. 2006 Investigations on the influence of boron on somatic embryogenesis of *Daucus carota*. Plant Cell Tissue Organ Cult. 84, 279-283.

MAUNEY J.R., CHAPPELL J. & WARD B.J. 1967 Effects of malic acid salts on growth of young cotton embryos *in vitro*. Bot. Gaz. 128, 198-200.

MAURO M.C., NEE C. & FALLOT J. 1986 Stimulation of somatic embryogenesis and plant regeneration from anther culture of *Vitis vinifera* cv. Cabernet-Sauvignon. Plant Cell Rep. 5, 377-380.

MAZÉ P. 1919 Recherche de physiologie végétale. Influence respective des éléments de la solution minérale sur la développement de mais. Ann. Inst. Pasteur 28, 21-46.

McCLENDON J.H. 1976 Elemental abundance as a factor in the origins of mineral nutrient requirements. J. Mol. Evol. 8, 175-195.

McCOWN B.H. & SELLMER J.C. 1987 General media and vessels suitable for woody plant culture. pp. 4-16 in Bonga and Durzan (eds.) 1987 *Cell and Tissue Culture in Forestry* Vol 1. *General Principles and Biotechnology*. Martinus Nijhoff Publishers, Dordrecht, Boston, Lancaster. ISBN 90-247-3430-4.

MENGEL K. & KIRKBY E.A. 1982 *Principles of Plant Nutrition*. 3rd Edition. Internat. Potash Institute, Bern, Switzerland.

MEYER Y. & ABEL W.O. 1975a Importance of the cell wall for cell division and in the activity of the cytoplasm in cultured tobacco protoplasts. Planta 123, 33-40.

MEYER Y. & ABEL W.O. 1975b Budding and cleavage division of tobacco mesophyll protoplasts in relation to pseudo-wall and wall formation. Planta 125, 1-13.

MIDDLETON W., JARVIS B.C. & BOOTH A. 1978 The boron requirement for root development in stem cuttings of *Phaseolus aureus* Roxb. New Phytol. 81, 287-297.

MILLER C.O. 1954 The influence of cobalt and sugar upon the elongation of etiolated pea stem segments. Plant Physiol. 29, 79-82.
MILLER C.O. 1961 A kinetin-like compound in maize. Proc. Natl. Acad. Sci. USA 47, 170-174.
MILLER C.O. 1963 Kinetin and kinetin-like compounds. pp. 194-202 in Linskens & Tracey (eds.). *Modern Methods of Plant Analysis*. Vol. 6. Springer-Verlag, Berlin.
MILLER L.R. & MURASHIGE T. 1976 Tissue culture propagation of tropical foliage plants. In Vitro 12, 797-813.
MILLERD A., SPENCER D., DUDMAN W.F. & STILLER M. 1975 Growth of immature pea cotyledons in culture. Aust. J. Plant Physiol. 2, 51-59.
MISHRA D. & KAR M. 1975 Nickel in plant growth and metabolism. Bot. Rev. 40, 395-452.
MITRA G.C., PRASAD R.N. & ROYCHOWDHURY A. 1976 Inorganic salts and differentiation of protocorms in seed-callus of an orchid and correlated changes in its free amino acid content. Ind. J. Exp. Biol. 14, 350-351.
MITSUKAWA N, OKUMURA S, SHIRANO Y, SATO S, KATO T, HARASHIMA S & SHIBATA D 1997 Overexpression of an *Arabidopsis thaliana* high-affinity phosphate transporter gene in tobacco cultured cells enhances cell growth under phosphate-limited conditions. Proc. Natl Acad. Sci. USA 94, 7098-7102.
MOHANTY B. & FLETCHER J.S. 1978 Influence of ammonium on the growth and development of suspension cultures of Paul's Scarlet rose. Physiol. Plant. 42, 221-225.
MOHANTY B. & FLETCHER J.S. 1980 Ammonium influence on nitrogen assimilating enzymes and protein accumulation in suspension cultures of Paul's Scarlet rose. Physiol. Plant. 48, 453-459.
MOLLER I.M. & PALMER J.M. 1981 The inhibition of exogenous NAD(P)H oxidation in plant mitochondria by chelators and mersalyl as a function of pH. Physiol. Plant. 53, 413-420.
MOLNAR S.J. 1988a High frequency of stable 5-methyl-DL-tryptophan resistance in *Brassica nigra* cell suspension cultures. Plant Cell Tissue Organ Cult. 15, 245-256.
MOLNAR S.J. 1988b Nutrient modifications for improved growth of *Brassica nigra* cell suspension cultures. Plant Cell Tissue Organ Cult. 15, 257-267.
MONNIER M. 1976 Culture *in vitro* de l'embryon immature de *Capsella bursa-pastoris* Moench. (L.). Rev. Cytol. Biol. Vég. 39, 1-120.
MONTEUUIS O., BON M.C. & BERTHON J.Y. 1987 Micro-propagation aspects of *Sequoiadendron giganteum* juvenile and mature clones. Acta Hortic. 212, 489-497.
MOORE H.M. & HIRSCH A.M. 1981 DNA synthesis and mitosis in boron-deficient and control sunflower root tips. Plant Physiol. 67, Suppl., 12.
MORIGUCHI T. & YAMAKI S. 1989 Prolonged storage of grape nodal culture using a low concentration of ammonium nitrate. HortScience 24, 372-373.
MOTT R.L., CORDTS J.M. & LARSON A.M. 1985 Nitrogen and growth regulator effects on shoot and root growth of soybean *in vitro*. pp. 336-337 in Henke R.R., Hughes, K.W., Constantin M.J., Hollaender A.A. and Wilson C.M. (eds.) *Tissue Culture in Forestry and Agriculture*. Plenum Press, New York, London.
MOYER B.G. & GUSTINE D.L. 1984 Regeneration of *Coronilla varia* L. (Crownvetch) plants from callus culture. Plant Cell Tissue Organ Cult. 3, 143-148.
MULLER A.J. & GRAFE R. 1978 Isolation and characterization of cell lines of *Nicotiana tabacum* lacking nitrate reductase. Mol. Gen. Genet. 161, 67-76.
MULLER A.J. & MENDEL R.R. 1982 Nitrate reductase-deficient tobacco mutants and the regulation of nitrate assimilation. pp. 233-234 in Fujiwara A. (ed.) 1982 (q.v.).
MULLINS K.V. 1987 Micropropagation of chestnut (*Castanea sativa* Mill.). Acta Hortic. 212, 525-530.
MURALIDHAR C.E. & MEHTA A.R. 1982 Clonal propagation of three ornamental plants through tissue culture methods. pp. 693-694 in A. (ed.) 1982 (q.v.).
MURASHIGE T. & SKOOG F. 1962 A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15, 473-497.
MURASHIGE T. 1974 Plant propagation through tissue cultures. Annu. Rev. Plant Physiol. 25, 135-166.
MURASHIGE T., SHABDE M.N., HASEGAWA P.M., TAKATORI F.H. & JONES J.B. 1972 Propagation of asparagus through shoot apex culture. I. Nutrient media for formation of plantlets. J. Am. Soc. Hortic. Sci. 97, 158-161.
NAGMANI R. & BONGA J.M. 1985 Embryogenesis in sub-cultured callus of *Larix decidua.* Can. J. For. Res. 15, 1088-1091.
NAS M.N. & READ P.E. 2004 A hypothesis for the development of a defined medium of higher plants and micropropagation of hazelnuts. Sci. Hortic. 101, 189-200.
NATO A., LAVERGNE D., FLIPO V. & HOARAU J. 1990 Are two localization sites of nitrate reductase responsible for the differential expression in tobacco cells during the growth cycle? pp. 349-354 in Nijkamp H.I.J., Van Der Plas I.H.W. and Van Aartrijk J. (eds.) *Progress in Plant Cellular and Molecular Biology.* Proc. VIIth Int. Cong. on Plant Tissue and Cell Culture. Amsterdam, The Netherlands. 24-29 June 1990. Kluwer Academic Publishers, Dortrecht, Netherlands.
NEALES T.F. 1959 The boron requirement of flax roots grown in sterile culture. J. Exp. Bot. 10, 426-436.
NEALES T.F. 1964 A comparison of the boron requirements of intact tomato plants and excised tomato roots grown in sterile culture. J. Exp. Bot. 15, 647-653.
NICKELL L.G. & MARETZKI A. 1969 Growth of suspension cultures of sugarcane cells in chemically defined media. Physiol. Plant. 22, 117-125.
NIRWAN R.S. & KOTHARI S.L. 2003 High copper levels improve callus induction and plant regeneration in *Sorghum bicolor* (L.) Moench. In Vitro Cell. Dev. Biol. Plant 39, 161-164.
NITSCH J.P. & NITSCH C. 1956 Auxin-dependent growth of excised *Helianthus* tissues. Am. J. Bot. 43, 839-851.
NITSCH J.P. 1969 Experimental androgenesis in *Nicotiana*. Phytomorph. 19, 389-404.
NOBÉCOURT P. 1937 Culture en serie des tissus végétaux sur milieux artificial. Compt. Rend. Acad. Sci. Paris 205, 521-523.
NORSTOG K. 1967 Studies on the survival of very small barley embryos in culture. Bull. Torrey Bot. Club 94, 223-229.
NORSTOG K. 1973 New synthetic medium for the culture of premature barley embryos. In Vitro 8, 307-308.
NUTI RONCHI V., CALIGO M.A., NOZZOLINI M. & LUCCARINI G. 1984 Stimulation of carrot somatic embryogenesis by proline and serine. Plant Cell Rep. 3, 210-214.
OCHATT S.J. & CASO O.H. 1986 Shoot regeneration from leaf mesophyll protoplasts of wild pear (*Pyrus communis* var. pyraster L.). J. Plant Physiol. 122, 243-249.
OCHATT S.J. & POWER J.B. 1988a Rhizogenesis in callus from conference pear (*Pyrus communis* L.) protoplasts. Plant Cell Tissue Organ Cult. 13, 159-164.
OCHATT S.J. & POWER J.B. 1988b Plant regeneration from mesophyll protoplasts of Williams' Bon Chretien (syn. Bartlett) pear (*Pyrus communis* L.). Plant Cell Rep. 7, 587-589.
ODHNOFF C. 1957 Boron deficiency and growth. Physiol. Plant. 10, 984-1000.

OERTLI J.J. & GRGUREVIC E. 1974 Effect of pH on the absorption of boron by excised embryos. Agron. J. 67, 278-280.

OHYAMA K. & NITSCH J.P 1972 Flowering haploid plants obtained from protoplasts of tobacco leaves. Plant Cell Physiol. 13, 229- 236.

ORCZYK W. & MALEPSZY S. 1985 *In vitro* culture of *Cucumis sativus* L. V. Stabilizing effect of glycine on leaf protoplasts. Plant Cell Rep. 4, 269-273

OSIFO F.O. 1988 Somatic embryogenesis in *Dioscorea*. J. Plant Physiol. 133, 378-380..

OZANNE P.G. 1958 Chlorine deficiency in soils. Nature 182, 1172-1173.

OZANNE P.G., WOOLLEY J.T. & BROYER T.C. 1957 Chlorine and bromine in the nutrition of higher plants. Austr. J. Biol. Sci. 10, 66-79.

OZIAS-AKINS P. & VASIL I.K. 1982 Plant regeneration from cultured immature embryos and inflorescences of *Triticum aestivum* L. (wheat): evidence for somatic embryogenesis. Protoplasma 110, 95-105.

PAIS M.S.S. & CASAL M. 1987 Propagation of the fern *Adiantum copillas-veneris* through tissue culture of the circinate part of young leaves. Acta Hortic. 212, 651-654.

PASQUALETTO P.-L., ZIMMERMAN R.H. & FORDHAM I. 1988 The influence of cation and gelling agent concentrations on vitrification of apple cultivars *in vitro*. Plant Cell Tissue Organ Cult. 14, 31-40

PÉRINET P., VALLÉE G. & TREMBLAY E.M. 1988 *In vitro* propagation of mature trees of *Alnus incana* (L.) Moench. Plant Cell Tiss. Organ Culture, 15, 85-89.

PEVALEK-KOZLINA B. & JELASKA S. 1987 Microclonal propagation of *Prunus avium* L. Acta Hortic. 212, 599-602.

PHILLIPS G.C. & COLLINS G.B. 1979 *In vitro* tissue culture of selected legumes and plant regeneration from callus cultures of red clover. Crop Sci. 19, 59-64.

PHILLIPS G.C. & COLLINS G.B. 1980 Somatic embryogenesis from cell suspension cultures of red clover. Crop Sci. 20, 323-326.

PIAGNANI C. & ECCHER T. 1988 Factors affecting the proliferation and rooting of chestnut *in vitro*. Acta Hortic. 227, 384-386.

PIERIK R.L.M. 1988 *In vitro* culture of higher plants as a tool in the propagation of horticultural crops. Acta Hortic. 226, 25-40.

PIERIK R.L.M., SPRENKELS P.A., VAN DER HARST R. & VAN DER MAYS Q.G. 1988 Seed germination and further development of plantlets of *Paphiopedilum ciliolare* Pfitz. *in vitro*. Scientia Hort. 34, 139-153.

POIRIER-HAMON S., RAO P.S. & HARADA H. 1974 Culture of mesophyll protoplasts and stem segments of *Antirrhinum majus* (snapdragon): growth and organization of embryoids. J. Exp. Bot. 25, 752-760.

POLACCO J.C. 1977a Is nickel a universal component of plant ureases? Plant Sci. Lett. 10, 249-255.

POLACCO J.C. 1977b Nitrogen metabolism in soybean tissue culture. II. Urea utilisation and urease synthesis require Ni^{2+}. Plant Physiol. 59, 827-830.

POLLARD A.S., PARR A.J. & LOUGHMAN C.L. 1977 Boron in relation to membrane function in higher plants. J. Exp. Bot. 28, 831-841.

POOVAIAH B.W. 1988 Molecular and cellular aspects of calcium action in plants. HortScience 23, 267-271.

PRICE H.J. & SMITH R.H. 1979a Somatic embryogenesis in suspension cultures of *Gossypium klotzschianum* Anderss. Planta 145, 305-307.

PRICE H.J. & SMITH R.H. 1979b Somatic embryogenesis in *Gossypium*. In Vitro 15, 177 (Abst. 45).

PRICE A.H., TAYLOR A., RIPLEY S.J., GRIFFITH A., TREWAVAS A.J. & KNIGHT M.R. 1994 Oxidative signals in tobacco increase cytosolic calcium. Plant Cell 6, 1301-1310.

PULLMAN G.S., MONTELLO P., CAIRNEY J., XU N. & FENG X. 2003 Loblolly pone (*Pinus taeda* L.) somatic embryogenesis: maturation improvements by metal analyses of zygotic and somatic embryos. Plant Sci. 164, 955-969.

QUOIRIN M. & LEPOIVRE P. 1977 Improved media for *in vitro* culture of *Prunus* sp. Acta Hortic. 78, 437-442.

RADOJEVIC L. 1988 Plant regeneration of *Aesculus hippocastanum* L. (Horse Chestnut) through somatic embryogenesis. J. Plant Physiol. 132, 322-326.

RAINS D.W. 1976 Mineral metabolism. pp. 561-597 in Bonner J. and Varner J.L. (eds.) 1976 *Plant Biochemistry*. Academic Press, New York.

RAMAGE C.M. & WILLIAMS R.R. 2002 Inorganic nitrogen requirements during shoot organogenesis in tobacco leaf discs. J. Exp. Bot. 53, 1437-1443.

RAMAGE C.M. & WILLIAMS R.R. 2002 Mineral nutrition and plant morphogenesis. In Vitro Cell. Dev. Biol. Plant 38, 116-124.

RANCH J. & WIDHOLM J. 1980 Studies on the use of toxic metabolic analogs for the isolation of nutritional variants from plant cell cultures. Plant Physiol. 65, Suppl., 37.

RANGA SWAMY N.S. 1958 Culture of nucellar tissue of Citrus *in vitro*. Experientia 14, 111-112.

RASHID A. & STREET H.E. 1973 The development of haploid embryoids from anther cultures of *Atropa belladonna* L. Planta 113, 263-270.

RAVEN J.A. 1986 Biochemical disposal of excess H^+ in growing plants? New Phytol. 104, 175-206.

REILLEY C.N. & SCHMID R.W. 1958 Chelometric titrations with potentiometric end point detection. Anal. Chem. 30, 947-953.

REINERT J. & WHITE P.R. 1956 The cultivation *in vitro* of tumor tissues and normal tissues of *Picea glauca*. Physiol. Plant. 9, 177-189.

REINERT J., TAZAWA M. & SEMENOFF S. 1967 Nitrogen compounds as factors of embryogenesis *in vitro*. Nature 216, 1215-1216.

REUVENY Z., DOUGALL D.K. & TRINITY P.M. 1980 Regula-tory coupling of nitrate and sulfate assimilation pathways in cultured tobacco cells. Proc. Natl. Acad. Sci. USA 77, 6670-6672.

RIGHETTI B., MAGNANINI E. & MACCAFERRI M. 1988 Ethylene and other volatile substances produced by *in vitro*-cultured *Prunus avium*. Acta Hortic. 227, 402-404.

RISSER P.G. & WHITE P.R. 1964 Nutritional requirements of Spruce tumour cells *in vitro*. Physiol. Plant. 17, 620-635.

ROEST S. & BOKELMANN G.S. 1975 Vegetative propagation of *Chrysanthemum morifolium* Ram. *in vitro*. Sci. Hortic. 3, 317-330.

ROMHELD V. & MARSCHNER H. 1986 Evidence for a specific uptake system for iron phytosiderophores in roots of grasses. Plant Physiol. 80, 175-180.

RUGINI E. & VERMA D.C. 1982 Micropropagation and cell suspensions of a difficult to propagate almond (*Prunus amygdalus* Batch) cultivar. pp. 741-742 in Fujiwara A. (ed.) 1982 (q.v.).

RUGINI E. 1984 *In vitro* propagation of some olive (*Olea europea sativa* L.) cultivars with different rootability, and medium development using analytical data from developing shoots and embryos. Sci. Hortic. 24, 123-124.

RUSSELL J.A. & McCOWN B.H. 1988 Recovery of plants from leaf protoplasts of hybrid-poplar and aspen clones. Plant Cell Rep. 7, 59-62.

SALISBURY F.B. 1959 Growth regulators and flowering. II. The cobaltous ion. Plant Physiol. 34, 598-604.
SANDERS D., BROWNLEE C. & HARPER J.F. 1999 Communicating with calcium. Plant Cell 11, 691-706.
SANDSTEDT R. & SKOOG F. 1960 Effects of amino acid components of yeast extract on the growth of tobacco tissue *in vitro*. Physiol. Plant. 13, 250-256.
SANGWAN R.S. & NORREEL B. 1975 Induction of plants from pollen grains of *Petunia* cultured *in vitro*. Nature 257, 222-224.
SARGENT P.A. & KING J. 1974 Investigations of growth-promoting factors in conditioned soybean root cells and in the liquid medium in which they grow: cytokinin-like compounds. Can. J. Bot. 52, 2459-2463.
SARKAR D., PANDEY S.K., SUD K.C. & CHANEMOUG-ASOUNDHARAM A. 2004 *In vitro* characterization of manganese toxicity in relation to phosphorus nutrition in potato (*Solanum tuberosum* L.). Plant Sci. 167, 977-986.
SAUNDERS J.W. & BINGHAM E.T. 1972 Production of alfalfa plants from callus tissue. Crop Sci. 12, 804-808.
SAUNDERS M.J. & HEPLER P.K. 1981 Localization of membrane-associated calcium following cytokinin treatment in *Funaria* using chlorotetracycline. Planta 152, 272-281.
SCHACHTMAN D.P., REID R.J. & AYLING S.M. 1998 Phosphorus uptake by plants: From soil to cell. Plant Physiol. 116, 447-453
SCHENK R.U. & HILDEBRANDT A.C. 1972 Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50, 199-204.
SCHERER P.A., MULLER E., LIPPERT H. & WOLFF G. 1988 Multielement analysis of agar and Gelrite impurities investigated by inductively coupled plasma emission spectrometry as well as physical properties of tissue culture media prepared with agar or gellan gum gelrite. Acta Hortic. 226, 655-658.
SCHOLTEN H.J. & PIERIK R.L.M. 1998 Agar as a gelling agent: Chemical and physical analysis. Plant Cell Rep. 17, 230-235.
SCHULZE D. 1988 *Saintpaulia ionantha* H. Wendl. – *In vitro* propagation and acclimatization in a commercial laboratory. Acta Hortic. 226, 619-622.
SCHWAMBACH J., FADANELLI C. & FETT-NETO A.G. 2005 Mineral nutrition and adventitious rooting in microcuttings of *Eucalyptus globulus*. Tree Physiol. 25, 487-494.
SCOWCROFT W.R. & ADAMSON J.A. 1976 Organogenesis from callus cultures of the legume *Stylosanthes hamata*. Plant Sci. Lett. 7, 39-42.
SHA L., McCOWN B.H. & PETERSON L.A. 1985 Occurrence and cause of shoot-tip necrosis in shoot cultures. J. Am. Soc. Hortic. Sci. 110, 631-634.
SHACKLOCK P.S., READ N.D. & TREWAVAS A.J. 1992 Cytosolic free calcium mediates red-light induced photomorphogenesis. Nature 358, 753-755.
SHARMA C.P. & SINGH S. 1990 Sodium helps overcome potassium deficiency effects on water relations of cauliflower. HortScience 25, 458-459.
SHEKHAWAT N.S. & GALSTON A.W. 1983 Mesophyll protoplasts of fenugreek (*Trigonella foenumgraecum*): Isolation, culture and shoot regeneration. Plant Cell Rep. 2, 119-121.
SHELTON B.B., EVANS G.F. & BOSS W.F. 1981 During embryogenesis, do cells exhibit polarity prior to morphological differentiation? Environ. Exp. Bot. 21, 426.
SHKOLNIK M. YA. 1984 Trace Elements in Plants. *Developments in Crop Science* 6. Elsevier Amsterdam, Oxford, New York, Tokyo.
SHORT K.C. & TORREY J.G. 1972 Cytokinin production in relation to the growth of pea-root callus tissue. J. Exp. Bot. 23, 1099-1105.
SINGH J.P. 1978 Effect of nitrogen sources on shoot bud differentiation of *Dioscorea deltoidea* Wall. callus culture. Biol. Plant. 20, 436-439.
SINGH M. & KRIKORIAN A.D. 1980 Chelated iron in culture media. Ann. Bot. 46, 807-809.
SINGHA S., OBERLEY G.H. & TOWNSEND E.C. 1987 Changes in nutrient composition and pH of the culture medium during *in vitro* shoot proliferation of crab apple and pear. Plant Cell Tissue Organ Cult. 11, 209-220.
SINGHA S., TOWNSEND E.C. & OBERLY, G.H. 1990 Relationship between calcium and agar on vitrification and shoot-tip necrosis of quince (*Cydonia oblonga* Mill.) shoots *in vitro*. Plant Cell Tissue Organ Cult. 23, 135-142.
SKOKUT T.A. & FILNER P. 1980 Slow adaptive changes in urease levels of tobacco cells cultured on urea and other nitrogen sources. Plant Physiol. 65, 995-1003.
SKOOG F. 1944 Growth and organ formation in tobacco tissue cultures. Am. J. Bot. 31, 19-24.
SMITH D.L. 1968 The growth of shoot apices and inflorescences of *Carex flacca* Schreb. in aseptic culture. Ann. Bot. 32, 361-370.
SMITH R.H. 1981 Phytohormone and primordial leaf effects on *Coleus blumei* apical domes. Plant Physiol. 67, Suppl., 118.
SOMMER A.L. & LIPMAN C.B. 1926 Evidence on the indispensable nature of zinc and boron for higher green plants. Plant Physiol. 1, 231-249.
SOMMER A.L. 1931 Copper as an essential for plant growth. Plant Physiol. 6, 339-345.
SONDAHL M.R. & SHARP W.R. 1977 High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. Z. Planzenphysiol. 81, 395-408.
STEWARD J.McD. & HSU C.L. 1977 *In ovulo* embryo culture and seedling development of cotton (*Gossypium hirsutum* L.). Planta 137, 113-117.
STREET H.E. 1966 The nutrition and metabolism of plant tissue and organ cultures. pp. 533-629 in Willmer E.N. *Cells and Tissues in Culture – Methods and Physiology.* Vol 3. Academic Press, London, New York.
STREET H.E. 1977 Laboratory organization. pp. 11-30 in Street H.E. (ed.) *Plant Tissue and Cell Culture.* Bot. Monographs Vol.11, Blackwell Scientific Public-ations. Oxford, London.
STREET H.E. 1979 Embryogenesis and chemically induced organogenesis. pp. 123-153 in Sharp W.R., Larsen P.O., Paddock E.F. and Raghavan V. (eds.) *Plant Cell and Tissue Culture*. Ohio State Univ. Press, Columbus
STREET H.E., McGONAGLE M.P. & McGREGOR S.M. 1952 Observations on the 'staling' of White's medium by excised tomato roots. II. Iron availability. Physiol. Plant. 5, 248-276.
STREET H.E. & McGREGOR S.M. 1952 The carbohydrate nutrition of tomato roots. III. The effects of external sucrose concentration on the growth and anatomy of excised roots. Ann. Bot. 16, 185-205.
SUBBARAO G.V., ITO O., BERRY W.L. & WHEELER R.M. 2003 Sodium – a functional plant nutrient. Crit. Rev. Plant Sci. 22, 391-416
SUEZAWA K., MATSUTA N., OMURA M. & YAMAKI S. 1988 Plantlet formation from cell suspensions of kiwi fruit (*Actinidia chinensis* Planch. var. *hispida*). Sci. Hortic. 37, 123-128.
SUGIURA A., TAO R., MURAYAMA H. & TOMANA T. 1986 *In vitro* propagation of Japanese persimmon. HortScience 21, 1205-1207.
SUNDQVIST C., BJORN L.O. & VIRGIN H.I. 1980 pp. 201-224 in Reinert J. (ed.) 1980 *Results and Problems in Cell Differentiation: 10. Chloroplasts*. Springer-Verlag Berlin, Heidelberg, New York.

SUZUKI A. & NATO A. 1982 Glutamate synthase isoforms in tobacco cultured cells. Immunological studies. pp. 239-240 in Fujiwara A. (ed.) 1982 (q.v.).

TAIRA T., HASKINS F.A. & GORZ H.J. 1977 Callus and suspension cultures of *Melilotus alba* tissues and cells. Crop Sci. 17, 407-411.

TAMAS I.A. 1987 Hormonal regulation of apical dominance. In: Davies, P.J. (ed.) *Plant hormones and their role in plant growth and development*. Martinus Nijhoff, Dordrecht. pp. 393-410.

TANADA T. 1978 Boron - key element in the actions of phytochrome and gravity? Planta 143, 109-111.

TANIMOTO S. & HARADA H. 1979 Influence of environmental and physiological conditions on floral bud formation of *Torenia* stem segments cultured *in vitro*. Z. Pflanzenphysiol. 95, 33-41.

TANIMOTO S. & HARADA H. 1981 Chemical factors controlling floral bud formation of *Torenia* stem segments cultured *in vitro*. I. Effects of mineral nutrients and sugars. Plant Cell Physiol. 22, 533-541.

TANIMOTO S. & HARADA H. 1982 Studies on floral initiation and development in *Torenia* stem segments cultured *in vitro*. pp. 155-156 in Fujiwara A. (ed.) 1982 (q.v.).

TANIMOTO S. & HARADA H. 1986 Involvement of calcium in adventitious bud initiation in *Torenia* stem segments. Plant Cell Physiol. 27, 1-10.

TANNER W. & BEEVERS H. 2001 Transpiration, a prerequisite for long-distance transport of minerals in plants? Proc. Natl. Acad. Sci. USA 98, 9443-9447.

TAUBCK K. 1942 Über die Lebensnotwendigkeit des Aluminiums fur Pteridophyten. Bot. Arch. 43, 291-295.

TAYLOR R.M. 1972 Germination of cotton (*Gossypium hirsutum* L.) pollen on an artificial medium. Crop Sci. 12, 243-244.

TAYLOR A.R., MANISON N.F.H., FERNANDEZ C., WOOD J. & BROWNLEE C. 1996 Spatial organization of calcium signalling involved in cell volume control in the *Fucus* rhizoid. Plant Cell 8, 2115-2031.

TEASDALE R.D. 1987 Micronutrients. pp. 17-49 in Bonga and Durzan (eds.) 1987 *Cell and Tissue Culture in Forestry* Vol 1. *General Principles and Biotechnology*. Martinus Nijhoff Publishers, Dordrecht, Boston, Lancaster. ISBN 90-247-3430-4.

TEASDALE R.D., DAWSON P.A. & WOOLHOUSE H.W. 1986 Mineral nutrient requirements of a loblolly pine (*Pinus taeda*) cell suspension culture. Plant Physiol. 82, 942-945.

TERRY N. 1977 Photosynthesis, growth and the role of chloride. Plant Physiol. 60, 69-75.

THIMANN K.V. & TAKAHASHI N. 1958 The action of chelating agents on growth of *Avena*. Plant Physiol. 33, Suppl., 33.

THOM M., MARETZKI A., KOMOR E. & SAKAI W.S. 1981 Nutrient uptake and accumulation by sugarcane cell cultures in relation to the growth cycle. Plant Cell Tissue Organ Cult. 1, 3-14.

THOMPSON J.F., MADISON J.T. & MUENSTER A.-M.E. 1977 *In vitro* culture of immature cotyledons of soya bean (*Glycine max* L.). Ann. Bot. 41, 29-39.

THORPE T.A. & MURASHIGE T. 1968 Some histochemical changes underlying shoot initiation in tobacco callus culture. Am. J. Bot. 55, 710.

THORPE T.A. & MURASHIGE T. 1970 Some histochemical changes underlying shoot initiation in tobacco callus cultures. Can. J. Bot. 48, 277-285.

TIFFIN L.O., BROWN J.C. & KRAUSS R.W. 1960 Differential absorption of metal chelate components by plant roots. Plant Physiol. 35, 362-367.

TIFFIN L.O. & BROWN J.C. 1961 Selective absorption of iron from iron chelates by soybean plants. Plant Physiol. 36, 710-714.

TIMMERS A.C.J., REISS H.D., BOHSUNG J., TRAXEL K. & SCHEL J.H.N. 1996 Localization of calcium during somatic embryogenesis of carrot (*Daucus carota* L.). Protoplasma 190, 107-118.

TORREY J.G. & REINERT J. 1961 Suspension cultures of higher plant cells in synthetic media. Plant Physiol. 36, 483-491.

TOURAINE B., GRIGNON N. & GRIGNON C. 1988 Charge balance in NO_3^--fed soybean. Estimation of K^+ and carboxylate recirculation. Plant Physiol. 88, 605-612.

TROLINDER N.L. & GOODIN J.R. 1988 Somatic embryogenesis in cotton (*Gossypium*). II. Requirements for embryo development and plant regeneration. Plant Cell Tissue Organ Cult. 12, 43-53.

TSUI C. 1948 The role of zinc in auxin synthesis in the tomato plant. Am. J. Bot. 35, 172-178.

UKAJI T. & ASHIHARA H. 1987 Effect of inorganic phosphate on the levels of amino-acids in suspension-cultured cells of *Catharanthus roseus*. Ann. Bot. 60, 109-114.

UMBECK P.F. & NORSTOG K. 1979 Effects of abscisic acid and ammonium ion on morphogenesis of cultured barley embryos. Bull. Torrey Bot. Club 106, 110-116.

VACIN E.F. & WENT F.W. 1949 Some pH changes in nutrient solutions. Bot. Gaz. 110, 605-613.

VALLES M. & BOXUS PH. 1987 Micropropagation of several *Rosa hybrida* L. cultivars. Acta Hortic. 212, 611-617.

VAN DER SALM T.P.M., VAN DER TOORN C.J.G., HÄNISH TEN CATE C.H., DUBOIS L.A.M., DE VRIES D.P. & DONS H.J.M. 1994 Importance of the iron chelate formula for micropropagation of *Rosa hybrida* L. 'Moneyway'. Plant Cell Tissue Organ Cult. 37, 73-77.

VAN WINKLE S.C., JOHNSON S., PULLMAN G.S. 2003 The impact of gelrite and activated carbon on the elemental composition of two conifer embryogenic tissue initiation media. Plant Cell Rep. 21, 1175 - 1182

VERMA D.C., LITVAY J.D., JOHNSON M.A. & EINSPHAR D.W.1982 Media development for cell suspensions of conifers. pp. 59-60 in Fujiwara A. (ed.) 1982 (q.v.).

WALKER K.A. & SATO S.J. 1981 Morphogenesis in callus tissue of *Medicago sativa*: the role of ammonium ion in somatic embryogenesis. Plant Cell Tissue Organ Cult. 1, 109-121.

WANG Z., ZENG X., CHEN C., WU H., LI Q., FAN G. & LU W. 1980 Induction of rubber plantlets from anther of *Hevea brasiliensis* Muell. Arg. *in vitro*. Chinese J. Trop. Crops 1, 16-26.

WANG Z., ZENG X., CHEN C., WU H., LI Q., FAN G. & LU W. 1981 Induction of plantlets from anthers of *Hevea in vitro*. Int. Rubber Res. Dev. Board Synposium, Haadyai 1981

WEBB D. & RIVERA M.E. 1981 Effects of NAA, BAP and L-glutamine on callus formation and organogenesis of *Zamia latifoliolata* embryos. Env. Exp. Bot. 21, 433-434 (Abst.).

WEINSTEIN L.H., MEISS A.N., UHLER R.L. & PURVIS E.R. 1956 Growth- promoting effects of ethylene-diamine tetra-acetic acid. Nature 178, 1188.

WEINSTEIN L.H., ROBBINS W.R. & PERKINS H.F. 1951 Chelating agents and plant nutrition. Science 120, 41-43.

WEISER C.J. & BLANEY L.T. 1960 The effects of boron on the rooting of English Holly cuttings. Proc. Am. Soc. Hortic. Sci. 75, 704-710.

WEISER C.J. 1959 Effect of boron on the rooting of clematis cuttings. Nature 183, 559-560.

WELANDER T. 1977 *In vitro* organogensis in explants from different cultivars of *Begonia hiemalis*. Physiol. Plant. 41, 142-145.

WELANDER T. 1987 *In vitro* culture of raspberry (*Rubus idaeus*) for mass propagation and virus elimination. Acta Hortic. 212, 610.

WETHERELL D.F. & DOUGALL D.K. 1976 Sources of nitrogen supporting growth and embryogenesis in cultured wild carrot tissue. Physiol. Plant. 37, 97-103.

WHITE M.C., DECKER A.M. & CHANEY R.L. 1981 Metal complexation in xylem fluid. I. Chemical composition of tomato and soybean stem exudate. Plant Physiol. 67, 292-300.

WHITE P.R. 1937 Separation from yeast of materials essential for growth of excised tomato roots. Plant Physiol. 12, 777-791.

WHITE P.R. 1938 Accessory salts in the nutrition of excised tomato roots. Plant Physiol. 13, 391-398.

WHITE P.R. 1939 Glycine in the nutrition of excised tomato roots. Plant Physiol. 14, 527-538.

WHITE P.R. 1942 Plant tissue cultures. Annu. Rev. Biochem. 11, 615-628.

WHITE P.R. 1943 *A Handbook of Plant Tissue Culture*. The Jacques Catlell Press, Lancaster, Pa.

WHITE P.R. 1954 *The Cultivation of Animal and Plant Cells*. 1st. edition. Ronald Press, New York.

WHITTINGTON W.J. 1959 The role of boron in plant growth. II. The effect on growth of the radicle. J. Exp. Bot. 10, 93-103.

WILLIAMS R.R. 1993 Mineral nutrition *in vitro* – A mechanistic approach. Aust. J. Bot. 41, 237-251.

WITTE C.P., TILLER S.A., TAYLOR M.A. & DAVIES H.V. 2002 Addition of nickel to Murashige and Skoog medium in plant tissue culture activates urease and may reduce metabolic stress. Plant Cell Tissue Organ Cult. 68, 103-104.

WOOD H.N. & BRAUN A.C. 1961 Studies on the regulation of certain essential biosynthetic systems in normal and crown-gall tumor cells. Proc. Natl. Acad. Sci. USA 47, 1907-1913.

WYN JONES R.G. & HUNT O.R. 1967 The function of calcium in plants. Bot. Rev. 33, 407-426.

YAMADA Y., ZHI-QI Y. & DING-TAI T. 1986 Plant regeneration from protoplast-derived callus of rice (*Oryza sativa* L.). Plant Cell Rep. 5, 85-88.

YOSHIDA F. & KOHNO H. 1982 Effects of media containing NH_4 as the sole nitrogen source on cultured cells of tobacco and rice. pp. 231-232 in Fujiwara A. (ed.) 1982 (q.v.).

YUSNITA S., GREEVE R.L. & KESTER R.T. 1990 Micropropagation of white Eastern Redbud (*Cercis canadensis* var. *alba*). HortScience 25, 1091 (Abst. 194).

ZAWADZKA M. & ORLIKOWSKA T.K. 2006 The influence of FeEDDHA on red raspberry cultures during shoot multiplication and adventitious regeneration from explants. Plant Cell Tissue Organ Cult. 85, 145-149.

ZELDIN E.L. & McCOWN B.H. 1986 Calcium gluconate can be used as a calcium source in plant tissue culture media. p. 57 in Somers D.A., Gegenbach B.G., Biesboer D.D., Hackett W.P. and Green C.E. (eds.) Abstracts VI Int. Cong. Plant Tissue and Cell Culture. Internat. Assoc. Plant Tiss. Cult. Minneapolis, Minn.

ZIMMERMAN R.H. 1981 Micropropagation of fruit plants. Acta Hortic. 120, 217-222.

ZINK M.W. & VELIKY I.A. 1979 Acid phosphatases of Ipomoea sp. cultured *in vitro*. 1. Influence of pH and inorganic phosphate on the formation of phosphatases. Can. J. Bot. 57, 739-753.

Chapter 4
The Components of Plant Tissue Culture Media II: Organic Additions, Osmotic and pH Effects, and Support Systems

1. ORGANIC SUPPLEMENTS

Growth and morphogenesis of plant tissue cultures can be improved by small amounts of some organic nutrients. These are mainly vitamins (including some substances that are not strictly animal vitamins), amino acids and certain undefined supplements. The amount of these substances required for successful culture varies with the species and genotype, and is probably a reflection of the synthetic capacity of the explant.

1.1. VITAMINS

Vitamins are compounds required by animals in very small amounts as necessary ancillary food factors. Absence from the diet leads to abnormal growth and development and an unhealthy condition. Many of the same substances are also needed by plant cells as essential intermediates or metabolic catalysts, but intact plants, unlike animals, are able to produce their own requirements. Cultured plant cells and tissues can however become deficient in some factors; growth and survival is then improved by their addition to the culture medium.

In early work, the requirements of tissue cultures for trace amounts of certain organic substances were satisfied by "undefined" supplements such as fruit juices, coconut milk, yeast or malt extracts and hydrolysed casein. These supplements can contribute vitamins, amino acids and growth regulants to a culture medium. The use of undefined supplements has declined as the need for specific organic compounds has been defined, and these have become listed in catalogues as pure chemicals.

1.2. THE DEVELOPMENT OF VITAMIN MIXTURES

The vitamins most frequently used in plant tissue culture media are thiamine (Vit. B_1), nicotinic acid (niacin) and pyridoxine (Vit. B_6) and apart from these three compounds, and *myo*-inositol, there is little common agreement about which other vitamins are really essential.

The advantage of adding thiamine was discovered almost simultaneously by Bonner (1937, 1938), Robbins and Bartley (1937) and White (1937). Nicotinic acid and pyridoxine appear, in addition to thiamine, in media published by Bonner (1940), Gautheret (1942) and White (1943b); this was following the findings of Bonner and Devirian (1939) that nicotinic acid improved the growth of isolated roots of tomato, pea and radish; and the papers of Robbins and Schmidt (1939a,b) which indicated that pyridoxine was also required for tomato root culture. These four vitamins; *myo*-inositol, thiamine, nicotinic acid, and pyridoxine are ingredients of Murashige and Skoog (1962) medium and have been used in varying proportions for the culture of tissues of many plant species (Chapter 3). However, unless there has been research on the requirements of a particular plant tissue or organ, it is not possible to conclude that all the vitamins which have been used in a particular experiment were essential.

The requirements of cells for added vitamins vary according to the nature of the plant and the type of culture. Welander (1977) found that Nitsch and Nitsch (1965) vitamins were not necessary, or were even inhibitory to direct shoot formation on petiole explants of *Begonia* x *hiemalis*. Roest and Bokelmann (1975) on the other hand, obtained increased shoot formation on *Chrysanthemum* pedicels when MS vitamins were present. Callus of *Pinus strobus* grew best when the level of inositol in MS medium was reduced to 50 mg/l whereas that of *P. echinata.* proliferated most rapidly when no inositol was present (Kaul and Kochbar, 1985).

Research workers often tend to adopt a 'belt and braces' attitude to minor media components, and add unusual supplements just to ensure that there is no missing factor which will limit the success of their experiment. Sometimes complex mixtures of as many as nine or ten vitamins have been employed.

Experimentation often shows that some vitamins can be omitted from recommended media. Although four vitamins were used in MS medium, later work at Professor Skoog's laboratory showed that the optimum rate of growth of tobacco callus tissue on MS salts required the addition of only *myo*-inositol and thiamine. The level of thiamine was increased four-fold over that used by Murashige and Skoog (1962), but nicotinic acid, pyridoxine and glycine

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(amino acid) were unnecessary (Linsmaier and Skoog, 1965). A similar simplification of the MS vitamins was made by Earle and Torrey (1965) for the culture of *Convolvulus* callus.

Soczck and Hempel (1988) found that in the medium of Murashige *et al.* (1974) devised for the shoot culture of *Gerbera jamesonii*, thiamine, pyridoxine and inositol could be omitted without any reduction in the rate of shoot multiplication of their local cultivars. Ishihara and Katano (1982) found that *Malus* shoot cultures could be grown on MS salts alone, and that inositol and thiamine were largely unnecessary.

1.3. SPECIFIC COMPOUNDS

***Myo*-inositol.** *Myo*-inositol (also sometimes described as *meso*-inositol or i-inositol) is the only one of the nine theoretical stereoisomers of inositol which has significant biological importance. Medically it has been classed as a member of the Vitamin B complex and is required for the growth of yeast and many mammalian cells in tissue culture. Rats and mice require it for hair growth and can develop dermatitis when it is not in the diet. *Myo*-inositol has been classed as a plant 'vitamin', but note that some authors think that it should be regarded as a supplementary carbohydrate, although it does not contribute to carbohydrate utilization as an energy source or as an osmoticum.

Historical use in tissue cultures. *Myo*-inositol was first shown by Jacquiot (1951) to favour bud formation by elm cambial tissue when supplied at 20-1000 mg/l. Necrosis was retarded, though the proliferation of the callus was not promoted. *Myo*-inositol at 100 mg/1 was also used by Morel and Wetmore (1951) in combination with six other vitamins for the culture of callus from the monocotyledon *Amorphophallus rivieri* (Araceae). Bud initials appeared on some cultures and both roots and buds on others according to the concentration of auxin employed. The vitamin was adopted by both Wood and Braun (1961) and Murashige and Skoog (1962) in combination with thiamine, nicotinic acid and pyridoxine in their preferred media fur the culture of *Catharanthus roseus* and *Nicotiana tabacum* respectively. Many other workers have since included it in culture media with favourable results on the rate of callus growth or the induction of morphogenesis. Letham (1966) found that *myo*-inositol interacted with cytokinin to promote cell division in carrot phloem explants.

Occurrence and biochemistry. Part of the growth promoting property of coconut milk is due to its *myo*-inositol content (Pollard *et al.,* 1961). Coconut milk also contains *scyllo*-inositol (Table 4.1). This can also promote growth but to a smaller extent than the *myo*-isomer (Pollard *et al.,* 1961). Inositol is a constituent of yeast extract (Steiner *et al.,* 1969; Steiner and Lester, 1972) and small quantities may also be contained in commercial agar (Wolter and Skoog, 1966). *Myo*-inositol is a natural constituent of plants and much of it is often incorporated into phosphatidyl-inositol which may be an important factor in the functioning of membranes (Jung *et al.,* 1972; Harran and Dickinson, 1978). The phosphatidylinositol cycle controls various cellular responses in animal cells and yeasts, but evidence of it playing a similar role in plants is only just being accumulated. Enzymes which are thought to be involved in the cycle have been observed to have activities in plants and lithium chloride (which inhibits *myo*-inositol-1-phosphatase and decreases the cycle) inhibits callus formation in *Brassica oleracea* (Bagga *et al.,* 1987), and callus growth in *Amaranthus paniculatus* (Das *et al.,* 1987). In both plants the inhibition is reversed by *myo*-inositol.

As the *myo*-inositol molecule has six hydroxyl units, it can react with up to six acid molecules forming various esters. It appears that inositol phosphates act as second messengers to the primary action of auxin in plants: phytic acid (inositol hexaphosphate) is one of these. Added to culture media it can promote tissue growth if it can serve as a source of inositol (Watanabe *et al.,* 1971). In some species, auxin can be stored and may be transported as IAA-*myo*-inositol ester (Chapter 5). *o*-Methyl-inositol is present in quite large quantities in legumes; inositol methyl ethers are known to occur in plants of several other families, although their function is unknown (Phillips and Smith, 1974).

The stimulatory effect of *myo*-inositol in plant cultures probably arises partly from the participation of the compound in biosynthetic pathways leading to the formation of the pectin and hemicelluloses needed in cell walls (Loewus *et al.,* 1962; Loewus, 1974; Loewus and Loewus, 1980; Harran and Dickinson, 1978; Verma and Dougall, 1979; Loewus and Loewus, 1980) and may have a role in the uptake and utilization of ions (Wood and Braun, 1961). In the experiments of Staudt (1984) mentioned below, when the $P0_4^{3-}$ content of the medium was raised to 4.41 mM, the rate of callus growth of cv. 'Aris' was

progressively enhanced as the *myo*- inositol in the medium was put up to 4000 mg/l. This result seems to stress the importance of inositol-containing phospholipids for growth.

Table 4.1. Substances identified as components of coconut milk (water) from mature green fruits and market-purchased fruits.

SUBSTANCE	QUANTITY/REFERENCE		SUBSTANCE	QUANTITY/REFERENCE	
	Mature green fruits	Mature fresh fruits		Mature green fruits	Mature fresh fruits
Amino acids (mg/l)			**Sugars (g/l)**		
Alanine	127.3 (14)	312 (13), 177.1 (14)	Sucrose	9.2 (14)	8.9 (14)
Arginine	25.6 (14)	133 (13), 16.8 (14)	Glucose	7.3 (14)	2.5 (14)
Aspartic acid	35.9 (14)	65 (13), 5.4 (14)	Fructose	5.3 (14)	2.5 (14)
Asparagine	10.1 (14)	ca.60 (13), 10.1 (14)	**Sugar alcohols (g/l)**		
γ-Aminobutyric acid	34.6 (14)	820 (13), 168.8 (14)	Mannitol		(1)
Glutamine acid	70.8 (14)	240 (13), 78.7 (14)	Sorbitol		15.0 (12), (17)
Glutamine	45.4 (14)	ca.60 (13), 13.4 (14)	*myo*-Inositol		0.1 (12), (17)
Glycine	9.7 (14)	13.9 (14)	*scyllo*-Inositol		0.5 (12), (17)
Histidine	6.3 (14)	Trace (13,14)	**Vitamins (mg/l)**		
Homoserine	-- (14)	5.2 (14)	Nicotinic acid		0.64 (4)
Hydroxyproline		Trace (13,14)	Pantolhenic acid		0.52 (4)
Lysine	21.4	65.8 (14)	Biotin, Riboflavin		0.02 (4)
Methionine	16.9 (14)	8 (13), Trace (14)	Riboflavin		0.01 (4)
Phenylalanine	-- (14)	12 (13), 10.2 (14)	Folic acid		0.003 (4)
Proline	31.9	97 (13), 21.6 (14)	Thiamine, pyridoxine		Trace (4)
Serine	45.3 (14)		**Growth substances (mg/l)**		
Typtophan		39 (13)	Auxin		0.07 (7), (28)
Threonine	16.2 (2)	44 (13), 26.3 (14)	Gibberellin		Yes (10,28)
Tyrosine	6.4 (14)	16 (13), 3.1 (14)	1,3-Diphenylurea		5.8 (8), (6,17)
Valine	20.6 (14)	27 (13), 15.1 (14)	Zeatin		(22,26)
Other nitrogenous compounds			Zeatin glucoside		(26)
Ammonium		(19)	Zeatin riboside		(20), (24), (25)
Ethanolamine		(19)	6-Oxypurine growth promoter		(27)
Dihydroxyphenyl alanine		(19)	Unknown cytokinin/s		6, (18) (22)
Inorganic elements (mg/100g dry wt.)			**Other (mg/l)**		
Potassium		312.0 (3)	RNA-polymerase		(23)
Sodium		105 (3)	RNA-phosphorus	20.0 (14)	35.4 (14)
Phosphorus		37.0 (3)	DNA-phosphorus	0.1 (14)	3.5 (14)
Magnesium		30.0 (3)	Uracil, Adenine		21
Organic acids (meq/ml)			Leucoanthocyanins		(11) (15,17)
Malic acid	34.3 (14)	12.0 (14)	Phyllococosine		(16)
Shikimic, Quinic and 2 unknowns	0.6 (14)	0.41 (2)	Acid Phosphatase		(5,9)
Pyrrolidone carboxylic acid	0.4 (14)	0.2 (14)	Diastase		(2)
Citric acid	0.4 (14)	0.3 (14)	Dehydrogenase		(5)
Succinic acid	-- (14)	0.3 (14)	Peroxidase		(5)
			Catalase		(5)

Numbered references (within brackets) in the above table are listed in Section 1.11 of this Chapter.

Activity in tissue cultures. Cultured plant tissues vary in their capacity for *myo*-inositol biosynthesis. Intact shoots are usually able to produce their own requirements, but although many unorganised tissues are able to grow slowly without the vitamin being added to the medium (Murashige, 1974) the addition of a small quantity is frequently found to stimulate cell division. The compound has been discovered to be essential to some plants. In the opinion of Kaul and Sabharwal (1975) this includes all monocotyledons, the media for which, if they do not contain inositol, need to be complemented with coconut milk, or yeast extract.

Fraxinus pennsylvanica callus had an absolute requirement for 10 mg/l *myo*-inositol to achieve maximum growth; higher levels, up to 250 mg/l had no further effect on fresh or dry weight yields (Wolter and Skoog, 1966). The formation of shoot buds on callus of *Haworthia* spp was shown to be dependent on the availability of *myo*-inositol (Kaul and Sabharwal, 1972, 1975). In a revised Linsmaier and Skoog (1965) medium [Staudt (1984) containing 1.84 mM PO_4^{3-}], callus tissue of *Vitis vinifera* cv 'Müller-Thurgau' did not require *myo*-inositol for growth, but that of *Vitis vinifera* x *V. riparia* cv. 'Aris' was dependent on it and the rate of growth increased as the level of *myo*-inositol was increased up to 250 mg/l (Staudt, 1984).

Gupta *et al.* (1988) found that it was essential to add 5 g/l *myo*-inositol to Gupta and Durzan (1985) DCR-1 medium to induce embryogenesis (embryonal suspensor masses) from female gametophyte tissue of *Pseudotsuga menziesii* and *Pinus taeda*. The concentration necessary seems insufficient to have acted as an osmotic stimulus (see section 3). *myo*-Inositol reduced the rate of proliferation in shoot cultures of *Euphorbia fulgens* (Zhang *et al.,* 1986).

Thiamine. Thiamine (Vit. B_1, aneurine) in the form of thiamine pyrophosphate, is an essential co-factor in carbohydrate metabolism and is directly involved in the biosynthesis of some amino acids. It has been added to plant culture media more frequently than any other vitamin. Tissues of most plants seem to require it for growth, the need becoming more apparent with consecutive passages, but some cultured cells are self sufficient. The maize suspension cultures of Polikarpochkina *et al.* (1979) showed much less growth in passage 2, and died in the third passage when thiamine was omitted from the medium.

MS medium contains 0.3 μM thiamine. That this may not be sufficient to obtain optimum results from some cultures is illustrated by the results of Barwale *et al.* (1986): increasing the concentration of thiamine-HCI in MS medium to 5 μM, increased the frequency with which zygotic embryos of *Glycine max* formed somatic embryos from 33% to 58%. Adding 30 μM nicotinic acid (normally 4 μM) improved the occurrence of embryogenesis even further to 76%. Thiamine was found to be essential for stimulating embryogenic callus induction in *Zoysia japonica*, a warm season turf grass from Japan (Asano *et al.,* 1996). It has also been shown to stimulate adventitious rooting of *Taxus* spp. (Chée, 1995).

There can be an interaction between thiamine and cytokinin growth regulators. Digby and Skoog (1966) discovered that normal callus cultures of tobacco produced an adequate level of thiamine to support growth providing a relatively high level of kinetin (ca. 1 mg/l) was added to the medium, but the tissue failed to grow when moved to a medium with less added kinetin unless thiamine was provided.

Sometimes a change from a thiamine-requiring to a thiamine-sufficient state occurs during culture (see habituation – Chapter 7). In rice callus, thiamine influenced morphogenesis in a way that depended on which state the cells were in. Presence of the vitamin in a pre-culture (Stage I) medium caused thiamine-sufficient callus to form root primordia on an induction (Stage II) medium, but suppressed the stimulating effect of kinetin on Stage II shoot formation in thiamine-requiring callus. It was essential to omit thiamine from the Stage I medium to induce thiamine-sufficient callus to produce shoots at Stage II (Inoue and Maeda, 1982).

1.4. OTHER VITAMINS

Pantothenic acid. Pantothenic acid plays an important role in the growth of certain tissues. It favoured callus production by hawthorn stem fragments (Morel, 1946) and stimulated tissue proliferation in willow and black henbane (Telle and Gautheret, 1947; Gautheret, 1948). However, pantothenic acid showed no effects with carrot, vine and Virginia creeper tissues which synthesize it in significant amounts (ca. 1 μg/ml).

Vitamin C. The effect of Vitamin C (L-ascorbic acid) as a component of culture media will be discussed in Chapter 12. The compound is also used during explant isolation and to prevent blackening.

Besides, its role as an antioxidant, ascorbic acid is involved in cell division and elongation, e.g., in tobacco cells (de Pinto *et al.,* 1999). Ascorbic acid (4-8 x 10^{-4} M) also enhanced shoot formation in both young and old tobacco callus. (Joy *et al.,* 1988). It speeded up the shoot-forming process, and completely reversed the inhibition of shoot formation by gibberellic acid in young callus, but was less effective in old callus. Clearly its action here was not as a vitamin.

Vitamin D. Some vitamins in the D group, notably vitamin D_2 and D_3 can have a growth regulatory effect on plant tissue cultures. Their effect is discussed in Chapter 7.

Vitamin E. The antioxidant activity of vitamin E (α-tocopherol) will be discussed in Chapter 12.

Other vitamins. Evidence has been obtained that folic acid slows tissue proliferation in the dark, while enhancing it in the light. This is probably because it is hydrolysed in the light to *p*-aminobenzoic acid (PAB). In the presence of auxin, PAB has been shown to have a weak growth-stimulatory effect on cultured plant tissues (de Capite, 1952a,b).

Riboflavin which is a component of some vitamin mixtures, has been found to inhibit callus formation but it may improve the growth and quality of shoots (Drew and Smith, 1986). Suppression of callus growth can mean that the vitamin may either inhibit or stimulate root formation on cuttings. Riboflavin has been shown to stimulate adventitious rooting on shoots of *Carica papaya* (Drew *et al.,* 1993), apple shoots (van der Krieken *et al.,* 1992) and *Eucalyptus globulus* (Trindade and Pais, 1997). It also enhances embryogenic callus induction in *Zoysia japonica* in association with cytokinins and thiamine (Asano *et al.,* 1996).

Glycine is occasionally described as a vitamin in plant tissue cultures: its use has been described in the section on amino acids.

Adenine. Adenine (or adenine sulphate) has been widely used in tissue culture media, but because it mainly gives rise to effects which are similar to those produced by cytokinins, it is considered in the chapter on cytokinins (Chapter 6).

Stability. Some vitamins are heat-labile; see the section on medium preparation in Volume 2.

1.5. UNDEFINED SUPPLEMENTS

Many undefined supplements were employed in early tissue culture media. Their use has slowly declined as the balance between inorganic salts has been improved, and as the effect of amino acids and growth substances has become better understood. Nevertheless several supplements of uncertain and variable composition are still in common use.

The first successful cultures of plant tissue involved the use of yeast extract (Robbins, 1922; White, 1934). Other undefined additions made to plant tissue culture media have been:

- meat, malt and yeast extracts and fibrin digest;
- juices, pulps and extracts from various fruits (Steward and Shantz, 1959; Ranga Swamy, 1963; Guha and Maheshwari, 1964, 1967), including those from bananas and tomatoes (La Rue, 1949);
- the fluids which nourish immature zygotic embryos;
- extracts of seedlings (Saalbach and Koblitz, 1978) or plant leaves (Borkird and Sink, 1983);
- the extract of boiled potatoes and corn steep liquor (Fox and Miller, 1959);
- plant sap or the extract of roots or rhizomes. Plant roots are thought to be the main site of cytokinin synthesis in plants (Chapter 6);
- protein (usually casein) hydrolysates (containing a mixture of all the amino acids present in the original protein). Casein hydrolysates are sometimes termed casamino acids: they are discussed in Chapter 3).

Many of these amendments can be a source of amino acids, peptides, fatty acids, carbohydrates, vitamins and plant growth substances in different concentrations. Those which have been most widely used are described below.

1.6. YEAST EXTRACT.

Yeast extract (YE) is used less as an ingredient of plant media nowadays than in former times, when it was added as a source of amino acids and vitamins, especially inositol and thiamine (Vitamin B_1) (Bonner and Addicott, l937; Robbins and Bartley, 1937). In a medium consisting only of macro- and micro-nutrients, the provision of yeast extract was often found to be essential for tissue growth (White, 1934; Robbins and Bartley, 1937). The vitamin content of yeast extract distinguishes it from casein hydrolysate (CH) so that in such media CH or amino acids alone, could not be substituted for YE (Straus and La Rue, 1954; Nickell and Maretzki, 1969). It was soon found that amino acids such as glycine, lysine and arginine, and vitamins such as thiamine and nicotinic acid, could serve as replacements for YE, for example in the growth of tomato roots (Skinner and Street, 1954), or sugar cane cell suspensions (Nickell and Maretzki, 1969).

The percentage of amino acids in a typical yeast extract is high (e.g. 7% amino nitrogen - Nickell and Maretzki, 1969; Bridson, 1978; Thom *et al.,* 1981), but there is less glutamic acid than in casein or other protein hydrolysate. Malt extract contains little nitrogen (ca. 0.5% in total).

Yeast extract has been typically added to media in concentrations of 0.1-1 g/l; occasionally 5, 10 and even 20 g/l (Morel and Muller, 1964) have been included. It normally only enhances growth in media containing relatively low concentrations of nitrogen, or where vitamins are lacking. Addition of 125-5000 mg/l YE to MS medium completely inhibited the growth of green callus of 5 different plants whereas small quantities added to Vasil and Hildebrandt (1966) THS medium (which contained 0.6 times the quantity of NO_3^- and NH_4^+ ions and unlike MS did not contain nicotinic acid or pyridoxine) gave more vigorous growth of carrot, endive and lettuce callus than occurred on MS. There was still no growth of parsley and tomato callus on THS medium: these tissues only grew well on unmodified MS (Vasil and Hildebrandt, 1966a,b,c).

Stage I media are sometimes fortified with yeast extract to reveal the presence of micro-organisms which may have escaped decontamination procedures: it is then omitted at later stages of culture.

Yeast extract has been shown to have some unusual properties which may relate to its amino acid content. It elicits phytoalexin accumulation in several plant species and in *Glycyrrhiza echinata* suspensions it stimulated chalcone synthase activity leading to the formation of narengin (Ayabe *et al.,* 1988). It also stimulated furomocoumarin production in *Glehnia littoralis* cell suspensions (Kitamura *et al.,* 1998). On Monnier (1976, 1978) medium 1 g/l yeast extract was found to inhibit the growth of immature zygotic embryos of *Linum*, an effect which, when 0.05 mg/1 BAP and 400 mg/l glutamine were added, induced the direct formation of adventitious embryos (Pretova and Williams, 1986).

Yeast extract is now purchased directly from chemical suppliers. In the 1930s and 1940s it was prepared in the laboratory. Brink *et al.* (1944) macerated yeast in water which was then boiled for 30 minutes and, after cooling, the starchy material was removed by centrifugation. However, Robbins and Bartley (1937) found that the active components of yeast could be extracted with 80% ethanol.

1.7. POTATO EXTRACT

Workers in China found that there was a sharp increase in the number of pollen plants produced from wheat anthers when they were cultured on an agar solidified medium containing only an extract of boiled potatoes, 0.1 mM FeEDTA, 9% sucrose and growth regulators. Potato extract alone or potato extract combined with components of conventional culture media (Chuang *et al.,* 1978) has since been found to provide a useful medium for the anther culture of wheat and some other cereal plants. For example, the potato medium was found to be better for the anther culture of spring wheat than the synthetic (N6) medium (McGregor and McHughen, 1990). Sopory *et al.* (1978) obtained the initiation of embryogenesis from potato anthers on potato extract alone and Lichter (1981) found it beneficial to add 2.5 g/l Difco potato extract to a medium for *Brassica napus* anther culture, but it was omitted by Chuong and Beversdorf (1985) when they repeated this work. We are not aware of potato extract being added to media for micropropagation, apart from occasional reports of its use for orchid propagation. Sagawa and Kunisaki (1982) supplemented 1 litre of Vacin and Went (1949) medium with the extract from 100g potatoes boiled for 5 minutes, and Harvais (1982) added 5% of an extract from 200g potatoes boiled in 1 litre water to his orchid medium. Of interest was the finding that potato juice treatment enabled *in vitro* cultures of *Doritaenopsis* (Orchidaceae) to recover from hyperhydricity (Zou, 1995).

1.8. MALT EXTRACT

Although no longer commonly used, malt extract seems to play a specific role in cultures of *Citrus*. Malt extract, mainly a source of carbohydrates, was shown to initiate embryogenesis in nucellar explants (Rangan *et al.,* 1968; Rangan, 1984). Several recent studies showed a role for the extract in the multiplication of *Citrus sinensis* somatic embryos (Das *et al.,* 1995), and in other *Citrus* spp. (Jumin, 1995), in the promotion of plantlet formation from somatic embryos derived from styles of different *Citrus* cultivars (De Pasquale *et al.,* 1994), and in somatic embryogenesis and plantlet regeneration from pistil thin cell layers of *Citrus* (Carimi *et al.,* 1999). Malt extract also promoted germination of early cotyledonary stage embryos arising from the *in vitro* rescue of zygotic embryos of sour orange (Carimi *et al.,* 1998). The extract is commercially available and used at a level of 0.5 – 1 g/l.

1.9. BANANA HOMOGENATE

Homogenised banana fruit is sometimes added to media for the culture of orchids and is often reported to promote growth. The reason for its stimulatory effect has not been explained. One suggestion mentioned earlier is that it might help to stabilise the pH of the medium. Pierik *et al.* (1988) found that it was slightly inhibitory to the germination of *Paphiopedilum ciliolare* seedlings but promoted the growth of seedlings once germination had taken place.

1.10. FLUIDS WHICH NOURISH EMBRYOS

The liquid which is present in the embryo sac of immature fruits of *Aesculus* (e.g. *A. woerlitzensis*) (Shantz and Steward, 1956, 1964; Steward and Shantz, 1959; Steward and Rao, 1970) and *Juglans regia* (Steward and Caplin, 1952) has been found to have a strong growth-promoting effect on some plant tissues cultured on simple media, although growth inhibition has occasionally been reported (Fonnesbech, 1972). Fluid from the immature female gametophyte of *Ginkgo biloba* (Steward and Caplin, 1952) and extracts from the female gametophyte of *Pseudotsuga menziesii* (Mapes and Zaerr, 1981) and immature *Zea mays* grains (less than two weeks after pollination) can have a similar effect. The most readily obtained fluid with this kind of activity is coconut milk (water).

1.11. COCONUT MILK/WATER

When added to a medium containing auxin, the liquid endosperm of *Cocos nucifera* fruits can induce plant cells to divide and grow rapidly. The fluid is most commonly referred to as coconut milk, although Tulecke *et al.* (1961) maintained that the correct English term is 'coconut water', because the term coconut milk also describes the white liquid obtained by grating the solid white coconut endosperm (the 'meat') in water and this is *not* generally used in tissue culture media. However, in this section, both terms are used.

Coconut milk was first used in tissue cultures by Van Overbeek *et al.* (1941, 1942) who found that its addition to a culture medium was necessary for the development of very young embryos of *Datura stramonium*. Gautheret (1942) found that coconut milk could be used to initiate and maintain growth in tissue cultures of several plants, and Caplin and Steward (1948) showed that callus derived from phloem tissue explants of *Daucus carota* roots grew much more rapidly when 15% coconut milk was added to a medium containing IAA. Unlike other undefined supplements to culture media (such as yeast extract, malt extract and casein hydrolysate) coconut milk has proved harder to replace by fully defined media. The liquid has been found to be beneficial for inducing growth of both callus and suspension cultures and for the induction of morphogenesis. Although commercial plant tissue culture laboratories (particularly those in temperate countries) would endeavour not to use this ingredient on account of its cost, it is still frequently employed for special purposes in research.

It is possible to get callus growth on coconut milk alone (Steward *et al.,* 1952), but normally it is added to a recognised medium. Effective stimulation only occurs when relatively large quantities are added to a medium; the incorporation of 10-15 percent by volume is quite usual. For instance, Burnet and Ibrahim (1973) found that 20% coconut milk (i.e. one-fifth of the final volume of the medium) was required for the initiation and continued growth of callus tissue of various *Citrus* species in MS medium; Rangan (1974) has obtained improved growth of *Panicum miliaceum* in MS medium using 2,4-D in the presence of 15% coconut milk. By contrast, Vasil and co-workers (e.g. Vasil and Vasil, 1981a,b) needed to add only 5% coconut milk to MS medium to obtain somatic embryogenesis from cereal callus and suspension cultures.

Many workers try to avoid having to use coconut milk in their protocols. It is an undefined supplement whose composition can vary considerably (Swedlund and Locy, 1988). However, adding coconut milk to media often provides a simple way to obtain satisfactory growth or morphogenesis without the need to work out a suitably defined formulation. Suggestions that coconut milk is essential for a particular purpose need to be treated with some caution. For instance, in the culture of embryogenic callus from root and petiole explants of *Daucus carota*, coconut milk could be replaced satisfactorily either by adenine or kinetin, showing that it did not contribute any unique substances required for embryogenesis (Halperin and Wetherell, 1964).

Preparation. Ready prepared coconut water (milk) can be purchased from some chemical suppliers, but the liquid from fresh nuts (obtained from the greengrocer) is usually perfectly adequate. One nut will usually yield at least 100 ml. The water is most simply drained from dehusked coconuts by drilling holes through two of the micropyles. Only normal uncontaminated water should be used and so

nuts should be extracted one by one, and the liquid endosperm from each examined to ascertain that it is unfermented before addition to a bulk supply. Water from green but mature coconuts may contain slightly different quantities of substances to that in the nuts purchased in the local market (Table 4.1) and has been said to be a more effective stimulant in plant media than that from ripe fruits, but Morel and Wetmore (1951) found to the contrary. Tulecke *et al.* (1961) discovered that the water from highly immature coconuts contained smaller quantities of the substances normally present in mature nuts.

References to composition of coconut water (numbers refer to citations in Table 4.1). (1) Dunstan (1906), (2) DeKruijff (1906), (3) McCance and Widdowson (1940), (4) Vandenbelt (1945), (5) Sadasivan (1951), (6) Shantz and Steward (1952), (7) Paris and Duhamet (1953), (8) Shantz and Steward (1955), (9) Wilson and Cutter (1955), (10) Radley and Dear (1958), (11) Steward and Shantz (1959), (12) Pollard *et al.* (1961), (13) Figures of Steward *et al.* (1961), given by Raghavan (1977), (14) Tulecke *et al.* (1961), (15) Steward and Mohan Ram (1961), (16) Kuraishi and Okumura (1961), (17) Steward (1963), (18) Zwar *et al.* (1963), (19) Steward *et al.* (1964), (20) Letham (1968), (21) Steward *et al.* (1969), (22) Zwar and Bruce (1970), (23) Mondal *et al.* (1972), (24) Letham (1974), (25) Van Staden and Drewes (1975), (26) Van Staden (1976), (27) Letham (1982), (28) Dix and Van Staden (1982).

Use of Coconut water. Coconut water is usually strained through cloth and deproteinized by being heated to 80-100°C for about 10 minutes while being stirred. It is then allowed to settle and the supernatant is separated from the coagulated proteins by filtration through paper. The liquid is stored frozen at -20°C. Borkird and Sink (1983) did not boil the water from fresh ripe coconuts, but having filtered it through several layers of cheesecloth, adjusted the pH to 10 with 2 N NaOH and then kept it overnight at 4°C. The following day the pH was re-adjusted to 7.0 with 5 N HCl, and the preparation was refiltered before being stored frozen at –20°C.

Some workers autoclave media containing coconut milk; others filter-sterilise coconut milk and add it to a medium after autoclaving has been carried out. Morel and Wetmore (1951) used filter sterilisation, but found that the milk lost its potency if stored sterile (but presumably unfrozen) for 3 months. Street (1977) advocated autoclaving coconut milk after it had been boiled and filtered; it was then stored at -20°C until required.

Active ingredients. The remarkable growth stimulating property of coconut milk has led to attempts to isolate and identify the active principles. This has proved to be difficult because the fractions into which coconut milk has been separated each possess only a small proportion of the total activity and the different components appear to act synergistically. Substances so far identified include amino acids, organic acids, nucleic acids, purines, sugars, sugar alcohols, vitamins, growth substances and minerals (Table 4.1). The variable nature of the product is illustrated in the table by the analytical results obtained by different authors.

Auxin activity. The liquid has been found to have some auxin activity which is increased by autoclaving, probably because any such growth substances exist in a bound form and are released by hydrolysis. But although coconut milk can stimulate the growth of some *in vitro* cultures in the absence of exogenous auxin, it normally contains little of this kind of growth regulator and an additional exogenous supply is generally required. In modern media, where organic compounds are often added in defined amounts, the main benefit from using coconut milk is almost certainly due to its providing highly active natural cytokinin growth substances.

Cytokinin activity. Coconut milk was shown to have cytokinin activity by Kuraishi and Okumura (1961) and recognised natural cytokinin substances have since been isolated [9-β-D-ribo-furanosyl zeatin (Letham, 1968); zeatin and several unidentified ones (Zwar and Bruce, 1970); N, N′-diphenyl urea (Shantz and Steward, 1955)] but the levels of these compounds in various samples of coconut milk have not been published. An unusual cytokinin-like growth promoter, 2-(3-methylbut-2-enylamino)-purin-6-one was isolated by Letham (1982).

Because coconut milk contains natural cytokinins, adding it to media often has the same effect as adding a recognised cytokinin. This means that a beneficial effect on growth or morphogenesis is often dependent on the presence of an auxin. Steward and Caplin (1951) showed that there was a synergistic action between 2,4-D and coconut milk in stimulating the growth of potato tuber tissue. Lin and Staba (1961) similarly found that coconut milk gave significantly improved callus growth on seedling explants of peppermint and spearmint initiated by 2,4-D, but only slightly improved the growth initiated by the auxin 2-BTOA (2-benziothiazoleoxyacetic acid). The occurrence of gibberellin-like substances in coconut milk has also been reported (Radley and Dear, 1958).

Suboptimum stimulation and inhibition. In cases where optimal concentrations of growth adjuvants have been determined, it has been found that the level of the same or analogous substances in coconut milk may be suboptimal. La Motte (1960) noted that 150 mg/l of tyrosine most effectively induced morphogenesis in tobacco callus cultures, but coconut milk added at 15% would provide only 0.96 mg/l of this substance (Tulecke *et al.,* 1961). Fresh and autoclaved coconut milk from mature nuts has proved inhibitory to growth or morphogenesis (Noh *et al.,* 1988) in some instances. It is not known which ingredients cause the inhibition but the growth of cultured embryos seems particularly liable to be prevented, suggesting that the compound responsible might be a natural dormancy-inducing factor such as abscisic acid. Van Overbeck *et al.* (1942, 1944) found that a factor was present in coconut milk which was essential for the growth of *Datura stramonium* embryos, but that heating the milk or allowing it to stand could lead to the release of toxic substances. These could be removed by shaking with alcohols or ether or lead acetate precipitation. Duhamet and Mentzer (1955) isolated nine fractions of coconut milk by chromatography, and found one of these to be inhibitory to cultured crown gall tissues of black salsify when more than 10-20% coconut milk was incorporated into the medium. Norstog (1965) showed that autoclaved coconut milk could inhibit the growth of barley embryos but that filter-sterilised milk was stimulatory. Coconut water inhibited somatic embryo induction in *Pinus taeda* (Li and Huang, 1996) and both autoclaved or filter-sterilized coconut milk inhibited the growth of wheat embryo-shoot apices (Smith, 1967).

2. ORGANIC ACIDS

Organic acids can have three roles in plant culture media:

- they may act as chelating agents, improving the availability of some micronutrients,
- they can buffer the medium against pH change,
- they may act as nutrients.

A beneficial effect is largely restricted to the acids of the Krebs' cycle. Dougall *et al.* (1979) found that 20 mM succinate, malate or fumarate supported maximum growth of wild carrot cells when the medium was initially adjusted to pH 4.5. Although 1 mM glutarate, adipate, pimelate, suberate, azelate or phthalate controlled the pH of the medium, little or no cell growth took place.

2.1. USE AS BUFFERS

The addition of organic acids to plant media is not a recent development. Various authors have found that some organic acids and their sodium or potassium salts stabilise the pH of hydroponic solutions (Trelease and Trelease, 1933) or *in vitro* media (Van Overbeek *et al.,* 1941, 1942; Arnow *et al.,* 1953), although it must be admitted that they are not as effective as synthetic biological buffers in this respect (see Section 5). Norstog and Smith (1963) discovered that 100 mg/l malic acid acted as an effective buffering agent in their medium for barley embryo culture and also appeared to enhance growth in the presence of glutamine and alanine. Malic acid, now at 1000 mg/l was retained in the improved Norstog (1973) Barley II medium. In the experiments of Schenk and Hildebrandt (1972) low levels of citrate and succinate ions did not impede callus growth of a wide variety of plants and appeared to be stimulatory in some species. The acids were also effective buffers between pH 5 and pH 6, but autoclaving a medium containing sodium citrate or citric acid caused a substantial pH increase..

2.1.1. Complexing with metals

Divalent organic acids such is citric, maleic, malic and malonic (depending on species) are found in the xylem sap of plants, where together with amino acids they can complex with metal ions and assist their transport (White *et al.,* 1981). These acids can also be secreted from cultured cells and tissues into the growth medium and will contribute to the conditioning effect. Ojima and Ohira (1980) discovered that malic and citric acids, released into the medium by rice cells during the latter half of a passage, were able to make unchelated ferric iron available, so correcting an iron deficiency.

2.1.2. Nutritional role

As explained in Chapter 3, adding Krebs' cycle organic acids to the medium can enhance the metabolism of NH_4^+. Gamborg and Shyluk (1970) found that some organic acids could promote ammonium utilisation and the incorporation of small quantities of sodium pyruvate, citric, malic and fumaric acids into the medium, was one factor which enabled Kao and Michayluk (1975) to culture *Vicia hajastana* cells at low density. Their mixture of

organic acid ions has been copied into many other media designed for protoplast culture. Cultures may not tolerate the addition of a large quantity of a free acid which will acidify the medium. For example, *Triticale* anther callus grew well on Chu *et al.* (1975) N6 medium supplemented with 35 mg/l of a mixture of sodium pyruvate, malic acid, fumaric acid, citric acid, but not when 100 mg/l was added (Chien and Kao, 1983). When organic anions are added to the medium from the sodium or potassium salts of an acid there are metallic cations to counterbalance the organic anions, and it seems to be possible to add larger quantities without toxicity. Five mM (1240 mg/l .3H_2O) potassium succinate enhanced the growth of cultured peach embryos (Ramming, 1990), and adding 15 mM (4052 mg/1 .4H_2O) sodium succinate to MS medium (while also increasing the sucrose content from 3% to 6%) increased the cell volume and dry weight of *Brassica nigra* suspensions by 2.7 times (Molnar, 1988).

Some plants seem to derive nutritional benefit from the presence of one particular organic acid. Murashige and Tucker (1969) showed that orange juice added to a medium containing MS salts promoted the growth of *Citrus* albedo callus. Malic and other Krebs' cycle acids also have a similar effect; of these, citric acid produces the most pronounced growth stimulation. A concentration of up to 10.4 mM can be effective (Goldschmidt, 1976; Einset, l978; Erner and Reuveni, 1981). Succulent plants, in particular those in the family Crassulaccae, such as *Bryophyllum* and *Kalanchoe* fix relatively large amounts of carbon dioxide during darkness, converting it into organic acids, of which malic acid is particularly important. The organic acids are metabolised during daylight hours. In such plants, malic acid might be expected to prove especially efficient in enhancing growth if added to a culture medium. Lassocinski (1985) has shown this to be the case in chlorophyll-deficient cacti of three genera. The addition of L-malic acid to the medium of Savage *et al.* (1979) markedly improved the rate of survival and vigour of small cacti or areoles.

Organic acid (citrate, lactate, succinate, tartrate, and oxalate) pretreatment of alfalfa callus dramatically decreased the growth of callus, but increased the subsequent yield of somatic embryos and embryo development, as well as conversion to plantlets (Nichol *et al.,* 1991). They suggested that the acids may act in the physiological selection for embryogenic callus, by inducing preferential growth of slower-growing-compact cell aggregates compared to the faster growing friable callus.

3. SUGARS -NUTRITIONAL AND REGULATORY EFFECTS

Carbohydrates play an important role in *in vitro* cultures as an energy and carbon source, as well as an osmotic agent. In addition, carbohydrate-modulated gene expression in plants is known (Koch, 1996). Plant gene responses to changing carbohydrate status can vary markedly. Some genes are induced, some are repressed, and others minimally affected. As in microorganisms, sugar-sensitive plant genes are part of an ancient system of cellular adjustment to critical nutrient availability. However, there is no evidence that this role of carbohydrate is important in normal growth and organized development in cell cultures.3.1. Sugars as energy sources

3.1.1. Carbohydrate autotrophy.

Only a limited number of plant cell lines have been isolated which are autotrophic when cultured *in vitro*. Autotrophic cells are capable of fully supplying their own carbohydrate needs by carbon dioxide assimilation during photosynthesis (Bergmann, 1967; Tandeau de Marsac and Peaud-Lenoel, 1972a,b; Chandler *et al.,* 1972; Anon, 1980; Larosa *et al.,* 1981). Many autotrophic cultures have only been capable of relatively slow growth (e.g. Fukami and Hildebrandt, 1967), especially in the ambient atmosphere where the concentration of carbon dioxide is low (see Chapter 12). However, since these early trials, very good progess is being made with photoautrophic shoot cultures and photo-autotrophic micropropagation is now possible (Kozai, 1991). Success is dependent on enriching the CO_2 concentrations in the vessels during the photoperiod, reducing or eliminating sugar from the medium, and optimising the *in vitro* environment.

Nevertheless, for the normal culture of either cells, tissues or organs, it is necessary to incorporate a carbon source into the medium. Sucrose is almost universally used for micropropagation purposes as it is so generally utilisable by tissue cultures. Refined white domestic sugar is sufficiently pure for most practical purposes. The presence of sucrose in tissue culture media specifically inhibits chlorophyll formation and photosynthesis (see below) making autotrophic growth less feasible.

3.2 ALTERNATIVES TO SUCROSE

3.2.1. Other Sugars.

The selection of sucrose as the most suitable energy source for cultures follows many comparisons between possible alternatives. Some of the first work of this kind on the carbohydrate nutrition of plant tissue was done by Gautheret (1945) using normal carrot tissue. Sucrose was found to be the best source of carbon followed by glucose, maltose and raffinose; fructose was less effective and mannose and lactose were the least suitable. The findings of this and other work is summarized in Table 4.2. Sucrose has almost invariably been found to be the best carbohydrate; glucose is generally found to support growth equally well, and in a few plants it may result in better *in vitro* growth than sucrose, or promote organogenesis where sucrose will not; but being more expensive than sucrose, glucose will only be preferred for micropropagation where it produces clearly advantageous results.

Multiplication of *Alnus crispa*, *A. cordata* and *A. rubra* shoot cultures was best on glucose, while that of *A. glutinosa* was best on sucrose (Tremblay and Lalonde, 1984; Tremblay *et al.,* 1984; Barghchi, 1988). Direct shoot formation from *Capsicum annum* leaf discs in a 16 h day required the presence of glucose (Phillips and Hubstenberger, 1985). Glucose is required for the culture of isolated roots of wheat (Furguson, 1967) and some other monocotyledons (Bhojwani and Razdan, 1983).

Table 4.2. The main sugars which can utilized by plants.
The value of as sugar for carbon nutrition is indicated by the size of the type.

SUGAR	Reducing Capacity	Products of hydrolytic/enzymatic breakdown
Monosaccharides		
Hexoses		
Glucose	Reducing sugar	None
Fructose	Reducing sugar	None
Galactose	Reducing sugar	None
Mannose	Reducing sugar	None
Pentoses		
Arabinose	Slow reduction	None
Ribose	Slow reduction	None
Xylose	Slow reduction	None
Disaccharides		
Sucrose	Non-reducing	Glucose, fructose
Maltose	Reducing sugar	Glucose
Cellobiose	Reducing sugar	Glucose
Trehalose	Non-reducing	Glucose
Lactose	Reducing sugar	Glucose, fructose
Trisaccharides		
Raffinose	Non-reducing	Glucose, galactose, fructose

Some other monosaccharides such as arabinose and xylose; disaccharides such as cellobiose, maltose and trehalose; and some polysaccharides; all of which are capable of being broken down to glucose and fructose (Table 4.2), can also sometimes be used as partial replacements for sucrose (Straus and LaRue, 1954; Sievert and Hildebrandt, 1965; Yatazawa *et al.,* 1967; Smith and Stone, 1973; Minocha and Halperin, 1974; Zaghmout and Torres, 1985). In *Phaseolus* callus, Jeffs and Northcote (1967) found that sucrose could be replaced by maltose and trehalose (all three sugars have an alpha-glucosyl radical at the non-reducing end), but not by glucose or fructose alone or in combination, or by several other different sugars. Galactose has been said to be toxic to most plant tissues; it inhibits the growth of orchids and other plants in concentrations as low as 0.01% (0.9 mM) (Ernst *et al.,* 1971; Arditti and Ernst, 1984). However, cells can become adapted and grown on galactose, e.g., sugar cane cells (Maretzski and Thom, 1978). The key was the induction of the enzyme galactose kinase, which converts galactose to galactose-1-phosphate. More recently, other reports on galactose use have appeared. It promoted callus growth in rugosa rose, but inhibited somatic embryogenesis (Kunitake *et al.,* 1993). Galactose

promoted early somatic embryo maturation stages in European silver fir (Schuller and Reuther, 1993). When used instead of sucrose, it improved rooting of *Annona squamosa* microshoots (Lemos and Blake, 1996). In addition, galactose has been found to reduce or overcome hyperhydricity in shoot cultures (Druart, 1988; see Volume 2). Fructose has also been reported to be effective in preventing hyperhydricity (Rugini *et al.,* 1987).

There are some situations where fructose supports growth just as well as sucrose or glucose (Steffen *et al.,* 1988) and occasionally it gives better results. Some orchid species have been reported to grow better on fructose than glucose (Ernst, 1967; Ernst *et al.,* 1971; Arditti, 1979). Fructose was the best sugar for the production of adventitious shoots from *Glycine max* cotyledonary nodes, especially if the concentration of nutrient salts supplied was inadequate (Wright *et al.,* 1986). Shoot and leaf growth and axillary shoot formation in *Castanea* shoot cultures was stimulated when sucrose was replaced by 30 g/l fructose. The growth of basal callus was reduced and it was possible to propagate from mature explants of *C. crenata*, although this was not possible on the same medium supplemented with sucrose (Chauvin and Salesses, 1988). However, fructose was reported to be toxic to carrot tissue if, as the sole source of carbon, it was autoclaved with White (1943a) A medium. When filter sterilized, fructose supported the growth of callus cultures which had a final weight 70% of those grown on sucrose (Pollard *et al.,* 1961).

Sucrose in culture media is usually hydrolysed totally, or partially, into the component monosaccharides glucose and fructose (see below) and so it is logical to compare the efficacy of combinations of these two sugars with that of sucrose. Kromer and Kukulczanka (1985) found that meristem tips of *Canna indica* survived better on a mixture of 25 g/l glucose plus 5 g/l fructose, than on 30 g/l sucrose. Germination of *Paphiopedilum* orchid seeds was best on a medium containing 5g/l fructose plus 5 g/l glucose; a mixture of 7.5 g/l of each sugar was optimal for further growth of the seedlings (Pierik *et al.,* 1988). In spite of its rapid hydrolysis to glucose and fructose, sucrose appears to have a specific stimulatory effect on embryo development in Douglas fir, that was not observed when it was replaced by the monosaccharides (Taber *et al.,* 1998).

The general superiority of sucrose over glucose for the culture of organised plant tissues such as isolated roots may be on account of the more effective translocation of sucrose to apical meristems (Butcher and Street, 1964). In addition, there could be an osmotic effect, because, from an equal weight of compound, a solution of glucose has almost twice the molarity of a sucrose solution, and will thus, in the absence of inversion of the disaccharide, induce a more negative water potential (see below).

Maltose. Plant species vary in their ability to utilise unusual sugars. For instance, although Gautheret (1945) could grow carrot callus on maltose, Mathes *et al.* (1973) obtained only minimal growth of *Acer* tissue on media supplemented with this sugar. Similarly, growth of soybean tissue on maltose is normally very slow, but variant strains of cells have been selected which can utilise it (Limberg *et al.,* 1979), perhaps because the new genotypes possessed an improved capacity for its active transport. Later studies have given a more prominent role to maltose as a component of tissue culture media. Maltose serves as both a carbon source and as an osmoticum. Compared to sucrose there is a slower rate of extracellular hydrolysis, it is taken up more slowly, and hydrolysed intracellularly more slowly. Maltose led to a substantial increase in somatic embryos from *Petunia* anthers (Raquin, 1983). It also led to an increase in callus induction and plantlet regeneration during *in vitro* androgenesis of hexaploid winter triticale and wheat (Karsai *et al.,* 1994). Maltose also increased callus induction in rice microspore culture, with an acceleration of initial cell divisions (Xie *et al.,* 1995). For barley microspore culture, the inclusion of maltose led to a higher frequency of green plants (Finnie *et al.,* 1989). Maltose has been reported to equal or surpass sucrose in supporting embryogenesis in a number of species, including carrot (Verma and Dougall, 1977; Kinnersley and Henderson, 1988), alfalfa (Strickland *et al.,* 1987), wild cherry (Reidiboym-Talleux *et al.,* 1999), *Malus* (Daigny *et al.,* 1996), *Abies* (Norgaard 1997) and loblolly pine (Li *et al.,* 1998). The number of plants regenerated from *indica* (Biswas and Zapata, 1993), and *japonica* (Jain *et al.,* 1997) rice varieties was also greater when protoplasts were cultured with maltose rather than sucrose. Transfer from a medium containing sucrose or glucose to one supplemented with maltose has been used by Stuart *et al.* (1986) and Redenbaugh *et al.* (1987) to enhance the conversion of alfalfa embryos. Similarly, maltose led to a much higher germination rate from asparagus somatic embryos than sucrose (Kunitake *et al.,* 1997).

Lactose. The disaccharide lactose has been detected in only a few plants. When added to tissue

culture media it has been found to induce the activity of β-galactosidase enzyme which can be secreted into the medium. The hydrolysis of lactose to galactose and glucose then permits the growth of *Nemesia strumosa* and *Petunia hybrida* callus, cucumber suspensions (Hess *et al.,* 1979; Callebaut and Motte, 1988), cotton callus and cell suspensions (Mitchell *et al.,* 1980), and Japanese morning glory callus (Hisajima and Thorpe, 1981). The key to lactose utilization in Japanese morning glory was not only the extracellular hydrolysis of this disaccharide, but the induction of galactose kinase, which prevented the accumulation of toxic galactose (Hisajima and Thorpe, 1985). Rodriguez and Lorenzo Martin (1987) found that adding 30 g/l lactose to MS medium instead of sucrose increased the number of shoots produced by a *Musa accuminata* shoot culture, but no new shoots were produced on subsequent subculture, although they were when sucrose was present.

In addition to lactose, plant cells have been shown to become adapted and then to grow on other galactose-containing oligosaccharides, including melibiose (Nickell and Maretzki, 1970; Gross *et al.,* 1981), raffinose (Wright and Northcote, 1972; Thorpe and Laishley, 1974; Gross *et al.,* 1981), and stachyose (Verma and Dougall, 1977; Gross *et al.,* 1981).

Corn syrups. Kinnersley and Henderson (1988) have shown that certain corn syrups can be used as carbon sources in plant culture media and that they may induce morphogenesis which is not provoked by supplementing with sucrose. Embryogenesis was induced in a 10-year old non-embryogenic cell line of *Daucus carota* and plantlets were obtained from *Nicotiana tabacum* anthers by using syrups. Those used contained a mixture of glucose, maltose, maltotriose and higher polysaccharides. Their stimulatory effect was reproduced by mixtures of maltose and glucose.

3.2.2. Sugar alcohols.

Sugar alcohols were thought not usually to be metabolised by plant tissues and therefore unavailable as carbon sources. For this reason, mannitol and sorbitol have been frequently employed as osmotica to modify the water potential of a culture medium. In these circumstances, sufficient sucrose must also be present to supply the energy requirement of the tissues. Adding either mannitol or sorbitol to the medium may make boron unavailable (See Chapter 3).

Mannitol was found to be metabolised by *Fraxinus* tissues (Wolter and Skoog, 1966). Later, studies with carrot and tobacco suspensions and cotyledon cultures of radiata pine showed that although mannitol was taken up very slowly, it was readily metabolized (Thompson *et al.,* 1986). Thus, this sugat alcohol is only of value as a short-term osmotic agent. In contrast, sorbitol is readily taken up and metabolized in some species. It has been found to support the growth of apple callus (Chong and Taper, 1972, 1974a,b) and that of other rosaceous plants (Coffin *et al.,* 1976), occasionally giving rise to more vigorous growth than can be obtained on sucrose. The ability of Rosaceae to use sorbitol as a carbon source is reported to be variety dependent. Albrecht (1986) found that shoot cultures of one crabapple variety required sorbitol for growth and would not grow on sucrose; another benefited from being grown on a mixture of sorbitol and sucrose and the growth of a third suffered if any sucrose was replaced by sorbitol. The apple rootstock 'Ottawa 3' produced abnormal shoots on sorbitol (Chong and Pua, 1985). Evidence is accumulating to show that sugar alcohols generally exhibit non-osmotic roles in regulating morphogenesis and metabolism in plants that do not produce polyols as primary photosynthetic products (Steinitz, 1999). In addition to being metabolised to varying degrees in heterotrophic cultures, such as tobacco, maize, rice, citrus and chichory, sugar alcohols stimulate specific molecular and physiological responses, where they apparently act as chemical signals.

The cyclic hexahydric alcohol *myo*-inositol does not seem to provide a source of energy (Smith and Stone, 1973) and its beneficial effect on the growth of cultured tissues when used as a supplementary nutrient must depend on its participation in biosynthetic pathways (see vitamins above).

3.2.3. Starch.

Cultured cells of a few plants are able to utilise starch in the growth medium and appear to do so by release of extracellular amylases (Nickell and Burkholder, 1950). Growth rates of these cultures are increased by the addition of gibberellic acid, probably because it increases the synthesis or secretion of amylase enzymes (Maretzki *et al.,* 1971, 1974).

3.3. HYDROLYSIS OF SUCROSE.

The remainder of this section on sugars is devoted to the apparent effects of sucrose concentration on cell differentiation and morphogenesis. Reports on

the subject should be tempered with the knowledge that some or all of the sucrose in the medium is liable to be broken down into its constituent hexose sugars, and that such inversion will also occur within plant tissues, where reducing sugar levels of at least 0.5 per cent are likely to occur (Helgeson *et al.,* 1972).

3.3.1. Autoclaving.

A partial hydrolysis of sucrose takes place during the autoclaving of media (Ball, 1953; Wolter and Skoog, 1966) the extent being greater when the compound is dissolved together with other medium constituents than when it is autoclaved in pure aqueous solution (Ferguson *et al.,* 1958). In a fungal medium, not dissimilar to a plant culture medium, Bretzloff (1954) found that sucrose inversion during autoclaving (15 min at 15 lbs/in^2) was dependent on pH in the following way:

pH 3.0	100%
pH 3.4	75%
pH 3.8	40%
pH 4.2	25%
pH 4.7	12.5%
pH 5.0	10%
pH 6.0	0%

These results suggest that the proportion of sucrose hydrolysed by autoclaving media at conventional pH levels (5.5-5.8) should be negligible. Most evidence suggests that this is not the case and that 10-15% sucrose can be converted into glucose and fructose. Cultures of some plants grow better in media containing autoclaved (rather than filter-sterilised) sucrose (Ball, 1953; Guha and Johri, 1966; Johri and Guha, 1963; Verma and Van Huystee, 1971; White, 1932) suggesting that the cells benefit from the availability of glucose and/or fructose. However, Nitsch and Nitsch (1956) noted that glucose only supported the growth of *Helianthus* callus if it had been autoclaved, and Romberger and Tabor (1971) that the growth of *Picea* shoot apices was less when the medium contained sucrose autoclaved separately in water (or sucrose autoclaved with only the organic constituents of the medium) than when all the constituents had been autoclaved together. They suggested that a stimulatory substance might be released when sugars are autoclaved with agar.

In other species there has been no difference between the growth of cultures supplied with autoclaved or filter sterilised sucrose (Mathes *et al.,* 1973) or growth has been less on a medium containing autoclaved instead of filter sterilised sucrose (Stehsel and Caplin, 1969).

3.3.2. Enzymatic breakdown.

Sucrose in the medium is also inverted into monosaccharides during the *in vitro* culture of plant material. This occurs by the action of invertase located in the plant cell walls (Burstrom, 1957; Yoshida *et al.,* 1973) or by the release of extracellular enzyme (King and Street, 1977). In most cultures, inversion of sucrose into glucose and fructose takes place in the medium; but, because the secretion of invertase enzymes varies, the degree to which it occurs differs from one kind of plant to another. After 28 days on a medium containing either 3 or 4% sucrose, single explants of *Hemerocallis* and *Delphinium* had used 20-30% of the sugar. Of that which remained in the medium which had supported *Hemerocallis*, about 45% was sucrose, while only 5% was sucrose in the media in which *Delphinium* had been grown. In both cases the rest of the sucrose had been inverted (Lumsden *et al.,* 1990).

The sucrose-inverting capacity of tomato root cultures was greatest in media of pH 3.6-4.7. Activity sharply declined in less acid media (Weston and Street, 1968). Helgeson *et al.* (1972) found that omission of IAA auxin from the medium in which tobacco callus was cultured, caused there to be a marked rise in reducing sugar due to the progressive hydrolysis of sucrose, both in the medium and in the tissue. A temporary increase in reducing sugars also occurred at the end of the lag phase when newly transferred callus pieces started to grow rapidly. It is interesting to note that cell wall invertase possessed catalytic activity *in situ*, whether or not tobacco tissue was grown on sucrose (Obata-Sasamoto and Thorpe, 1983).

In cultures of some species, uptake of sugar may depend on the prior extracellular hydrolysis of sugar. This is the case in sugar cane (Komor *et al.,* 1981); and possibly also in *Dendrobium* orchids (Hew *et al.,* 1988) and carrot (Kanabus *et al.,* 1986). Nearly all the sucrose in suspension cultures of sugar cane and sugar beet was hydrolysed in 3 days (Zamski and Wyse, 1985) and *Daucus carota* suspensions have been reported to hydrolyse all of the sucrose in the medium (Thorpe, 1982) into the constituent hexoses within 3 days (Kanabus *et al.,* 1986) or within 24 h (Dijkema *et al.,* 1990). However, most species can take up sucrose directly as was shown through studies with asymetrically labeled ^{14}C-sucrose (Parr and Edelman, 1975), and metabolise it intracellularly. Within the cell, soluble invertase, sucrose phosphate synthetase and sucrose synthetase serve to hydrolyse sucrose (Thorpe, 1982). Thus, in *Acer* (Copping and

Street, 1972) the soluble invertase activity paralleled growth rate, while in tobacco (Thorpe and Meier, 1973) and Japanese morning glory (Hisajima *et al.,* 1978) sucrose synthetase was more important. In the last species, the change in activity of sucrose synthetase was greater than that of sucrose phosphate synthetase, an enzyme not extensively examined in cultured cells.

The growth of shoots from non-dormant buds of mulberry is not promoted by sucrose, only by maltose, glucose or fructose. Even though mulberry tissue hydrolysed sucrose into component monosaccharides, shoots did not develop. In the presence of 3% fructose, sucrose was actually inhibitory to shoot development at concentrations as low as 0.2% (Oka and Ohyama, 1982).

3.4. UPTAKE.

The uptake of sugar molecules into plant tissues appears to be partly through passive permeation and partly through active transport. The extent of the two mechanisms may vary. Active uptake is associated with the withdrawal of protons (H^+) from the medium. Charge compensation is effected by the excretion of a cation (H^+ or K^+) (Komor *et al.,* 1977, 1981). Glucose was taken up preferentially by carrot suspensions during the first 7 days of a 14 day passage; fructose uptake followed during days 7-9 (Dijkema *et al.,* 1990). At concentrations below 200 mM, glucose was taken up more rapidly into strawberry fruit discs and protoplasts than either sucrose or fructose (Scott and Breen, 1988).

3.5. EFFECTIVE CONCENTRATIONS.

In most of the comparisons between the nutritional capabilities of sugars discussed above, the criterion of excellence has been the most rapid growth of unorganised callus or suspension-cultured cells. For this purpose 2-4% sucrose w/v is usually optimal. Similar concentrations are also used in media employed for micropropagation, but laboratories probably pay insufficient attention to the effects of sucrose on morphogenesis (see below) and plantlet development. Sucrose levels in culture media which result in good callus growth may not be optimal for morphogenesis, and either lower or higher levels may be more effective.

The optimum concentration of sucrose to induce morphogenesis or growth differs between different genotypes, sometimes even between those which are closely related. For instance, Damiano *et al.* (1987) found that the concentration of sucrose necessary to produce the best rate of shoot proliferation in *Eucalyptus gunnii* shoot cultures varied between clones. The influence of sucrose concentration on direct shoot formation from *Chrysanthemum* explants varied with plant cultivar (Fig 4.1).

Experiments of Molnar (1988) have shown that the optimum level of sucrose may depend upon the other amendments added to a culture medium. The most rapid growth of *Brassica nigra* suspensions on one containing MS salts (but less iron and B5 vitamins) occurred when 2% sucrose was added. However, when it was supplemented with 1-4 g/l casein hydrolysate or a mixture of 3 defined amino acids, growth was increased on up to 6% sucrose. A similar result was obtained if, instead of the amino acids, 15 mM sodium succinate was added. There was an extended growth period and the harvested dry weight of the culture was 2.8 times that on the original medium with 2% sucrose.

The level of sucrose in the medium may have a direct effect on the type of morphogenesis. Thus, sucrose (87 mM) favored organogenesis, while a higher level (350 mM) favoured somatic embryogenesis from immature zygotic embryos of sunflower (Jeannin *et al.,* 1995). *In vitro* minicrowns of asparagus developed short, thickened storage roots at high frequencies when the sucrose concentration in the medium was increased to 6% (Conner and Falloon, 1993). Lower sucrose concentrations, even with the addition of non- or poorly metabolised carbohydrates, such as cellobiose, maltose, mannose, melibiose and sorbitol produced thin fibrous roots, indicating that the additional sucrose was nutritional rather than osmotic.

The respiration rate of cultured plant tissues rises as the concentration of added sucrose or glucose is increased. In wheat callus, it was found to reach a maximum when 90 g/1 (0.263 M) was added to the medium, even though 20 g/l produced the highest rate of growth and number of adventitious shoots (Galiba and Erdei, 1986). The uptake of inorganic ions can be dependent on sugar concentration and the benefit of adding increased quantities of nutrients to a medium may not be apparent unless the amount of sugar is increased at the same time (Gamborg *et al.,* 1974).

3.5.1. Cell differentiation

Formation of vascular elements. Although sugars are clearly involved in the differentiation of xylem and phloem elements in cultured cells, it is still uncertain whether they have a regulatory role apart from providing a carbon energy source necessary for

active cell metabolism. Sucrose is generally required to be present in addition to IAA before tracheid elements are differentiated in tissue cultures. The number of both sieve and xylem elements formed [and possibly the proportion of each kind - Wetmore and Rier (1963); Rier and Beslow (1967)] depends on sucrose concentration (Aloni, 1980). In *Helianthus tuberosus* tuber slices, although sucrose, glucose and trehalose were best for supporting cell division and tracheid formation, maltose was only a moderately effective carbon source (Minocha and Halperin, 1974). Shininger (1979) has concluded that only carbohydrates which enable significant cell division are capable of promoting tracheary element formation. The occurrence of lignin in cultured cells is not invariably associated with thickened cell walls. Sycamore suspension cultures produced large amounts of lignin when grown on a medium with abnormally high sucrose (more than 6%) and 2,4-D levels. It was deposited within the cells and released into the medium (Carceller *et al.,* 1971).

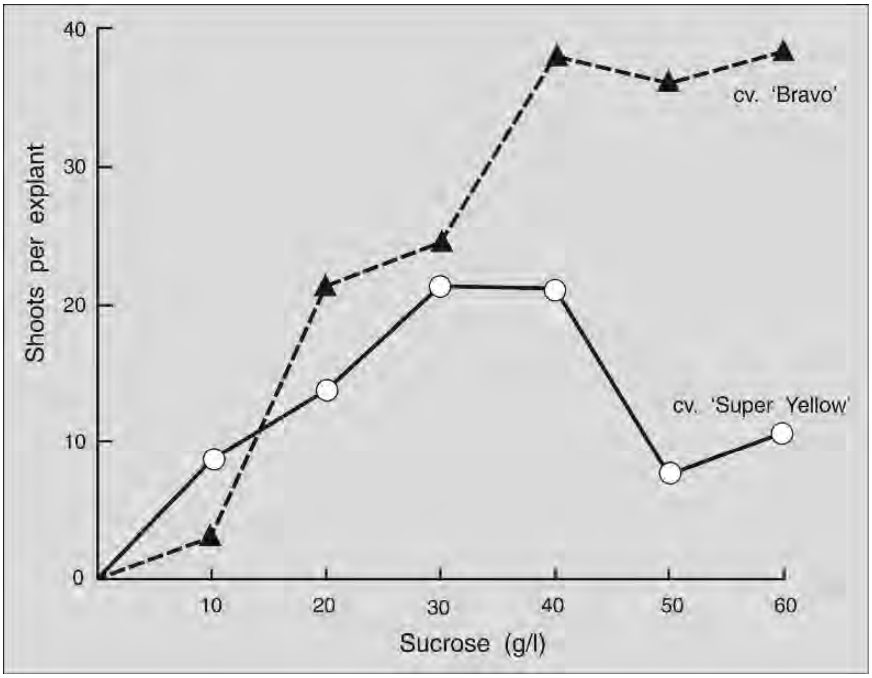

Fig. 4.1 The effect of sucrose concentration on direct adventitious shoot formation from flower pedicels of two chrysanthemum cultivars [from data of Roest and Bokelmann, 1975].

Chlorophyll formation. Levels of sucrose normally used to support the growth of tissue cultures are often inhibitory to chlorophyll synthesis (Rier and Chen, 1964; Edelman and Hanson, 1972) but the degree of inhibition does vary according to the species of plant from which the tissue was derived. In experiments of Hildebrandt *et al.* (1963) and Fukami and Hildebrandt (1967) for example, carrot and rose callus had a high chlorophyll content on Hildebrandt *et al.* (1946) tobacco medium with 2-8% sucrose; but tissue of endive, lettuce and spinach only produced large amounts of the pigment on a medium with no added sucrose (although a small amount of sugar was probably supplied by 15-16% coconut milk).

Cymbidium protocorms contain high chlorophyll levels only if they are cultured on media containing 0.2-0.5% sucrose. Their degree of greening declines rapidly when they are grown on sucrose concentrations higher than this (Vanséveren-Van Espen, 1973). Likewise, orchid protocorm-like bodies will not become green and cannot develop into plantlets if sucrose is present in the medium beyond the stage of their differentiation from the explants. Where added sucrose does reduce chlorophyll formation, it is thought that the synthesis of 5-aminolaevulinic acid (ALA - a precursor of the porphyrin molecules of which chlorophyll is composed) is reduced due to an inhibition of the activity of the enzyme ALA synthase (Pamplin and

Chapman, 1975). Sugars apart from sucrose are not inhibitory (Edelman and Hanson, 1972; El Hinnawiy, 1974). It has been said that cells grown on sucrose for prolonged periods may permanently lose the ability to synthesise chlorophyll (Van Huystee, 1977). Plastids become converted to amyloplasts packed with starch and this may change the expression of plastid DNA (Gunning and Steer, 1975) or result in a reduction of plastid RNA (Rosner *et al.,* 1977).

A small amount of photosynthesis may be carried out by cultured shoots providing they are not maintained on media containing a high concentration of sucrose. Photosynthesis increased in *Rosa* shoot cultures when they were grown initially on 20 or 40 g/l sucrose which was decreased to 10 g/l in successive subcultures (Langford and Wainwright, 1987). An increase in photosynthesis occurs when sucrose is omitted from the medium in which rooted plantlets are growing (Short *et al.,* 1987), but these treatments are not successful in ensuring a greater survival of plantlets when they are transferred *extra vitrum*. A more recent study also showed the relative contribution of autotrophic and heterotrophic carbon metabolism in cultured potato plants (Wolf *et al.,* 1998). With 8% sucrose in the medium 90% of the tissue carbon was of heterotrophic origin in light-grown plants; while on 3% sucrose, only 50% was of heterotrophic origin.

3.6 STARCH ACCUMULATION AND MORPHOGSNESIS

3.6.1. Starch deposition preceeding morphogenesis.

Cells of callus and suspension cultures commonly accumulate starch in their plastids and it is particularly prevalent in cells at the stationary phase. Starch in cells of rice suspensions had different chemical properties to that in the endosperm of seeds (Landry and Smyth, 1988). In searching for features which might be related to later morphogenetic events in *Nicotiana* callus, Murashige and co-workers (Murashige and Nakano, 1968; Thorpe and Murashige, 1968a, b) noticed that starch accumulated preferentially in cells sited where shoot primordia ultimately formed. The starch is produced from sucrose supplied in the culture medium (Thorpe *et al.,* 1986). As a result of this work, it was suggested that starch accumulation might be a prerequisite of morphogenesis. In tobacco, starch presumably acts as a direct cellular reserve of the energy required for morphogenesis, because it disappears rapidly as meristenoids and shoot primordia are formed (Thorpe and Meier, 1974, 1975). Morphogenesis is an energy-demanding process and callus maintained on a shoot-inducing medium does have a greater respiration rate than similar tissue kept on a non-inductive medium (Thorpe and Murashige, 1970; Thorpe and Meier, 1972). Organ-forming callus of tobacco has been found to accumulate starch prior to shoot or root formation, whereas callus not capable of morphogenesis did not do so (Kavi Kishor and Mehta, 1982).

3.6.2. Morphogenesis without starch deposition

Cells of other plants which become committed to initiate organs do not necessarily accumulate starch as a preliminary to morphogenesis and it seems likely that the occurrence of this phenomenon is species-related. The deposition of starch was observed as an early manifestation of organogenesis in *Pinus coulteri* embryos (Patel and Berlyn, 1983), but not in those of *Picea abies* (Von Arnold, 1987). Although zygotic embryos of the latter species immediately began to accumulate starch (particularly in the chloroplasts of cells in the cortex) when they were placed on a medium containing sucrose. It was never observed in meristematic cells from which adventitious buds developed (Von Arnold, 1987). However, if a major role for the accumulation of starch prior to the initiation of organized development is for energy production, this role would be satisfied by the lipid reserves in zygotic embryos of conifers (Thorpe, 1982). Indeed, the rapid and nearly linear degradation of triglycerides during the period of high respiration during shoot initiation in excised cotyledons of radiata pine (Biondi and Thorpe, 1982; Douglas *et al.,* 1982) would support this view.

Meristematic centres in bulb scales of *Nerine bowdenii* can be detected as groups of cells from which starch is absent (Grootaarts *et al.,* 1981). Starch was not accumulated in caulogenic callus of *Rosa persica* x *R. xanthina* initiated from recently-initiated shoot cultures, but cells did accumulate starch when the shoot forming capacity of the callus was lost after more than three passages (Lloyd *et al.,* 1988). Callus derived from barley embryos was noted to accumulate starch very rapidly and this was accompanied by a reduction in osmotic pressure within the cells (Granatek and Cockerline, 1978). Gibberellic acid, which in this plant could be used to induce shoot formation, brought about an increase in cell osmolarity.

There are some further examples where a diminution of the amount of stored carbohydrate in cultured tissues has restored or improved their

organogenetic capacity. A salt-tolerant line of alfalfa cells which showed no ability for shoot regeneration after three and a half years in culture on 3% sucrose was induced to form shoots and plantlets by being cultured for one passage of 24 days on 1% sucrose, before being returned to a medium containing 3% sucrose and a high 2,4-D level (Rains *et al.,* 1980, and personal communication). Cells which in 3% sucrose were full of starch became starch-depleted during culture on a lower sucrose level. The number of somatic embryos formed by embryogenic 'Shamouti' orange callus, was increased when sucrose was omitted from the medium for one passage, before being returned to Murashige and Tucker (1969) medium with 5-6% sucrose (Kochba and Button, 1974).

3.6.3. Unusual sugars

In some plants, unusual sugars are able to regulate morphogenesis and differentiation. Galactose stimulates embryogenesis in *Citrus* cultures (Kochba *et al.,* 1978) and can enhance the maturation of alfalfa embryos. Callus of *Cucumis sativus* grew most rapidly on raffinose and was capable of forming roots when grown on this sugar; somatic embryos were only differentiated when the callus was cultured on sucrose (88-175 mM), but if a small amount of stachyose (0.3 mM) was added to 88 mM sucrose the callus produced adventitious shoots instead (Kim and Janick, 1989). Stachyose is the major translocated carbohydrate in cucurbits.

4. OSMOTIC EFFECT OF MEDIA INGREDIENTS

Besides having a purely nutritive effect, solutions of inorganic salts and sugars, which compose tissue culture media, influence plant cell growth through their osmotic properties. A discussion is most conveniently accommodated at this point, as many of the papers published on the subject stress the osmotic effects of added sugars.

4.1. OSMOTIC AND WATER POTENTIALS: A GENERAL INTRODUCTION

Water movement into and out of a plant cell is governed by the relative concentrations of dissolved substances in the external and internal solutions, and by the pressure exerted by its restraining cell wall. The manner of defining the respective forces has changed in recent years, and as both old and new terminology are found in the tissue culture literature, the following brief description may assist the reader. More detailed explanations can be found in many text books on plant physiology.

In the older concept, cells were considered to take up water by suction (i.e. by exerting a negative pressure) induced by the osmotically active concentration of dissolved substances within the cell. The suction force or suction pressure (SP) was defined as that resulting from the osmotic pressure of the cell sap (OP_{cs}) minus the osmotic pressure of the external solution (OP_{ext}), and the pressure exerted on, and stretching the cell wall, turgor pressure (TP) - so called because it is at a maximum when the cells are turgid. This may be represented by the equations:

$SP = (OP_{cs} - OP_{ext}) – TP$ or

$SP = OP_{cs} - (OP_{ext} + TP)$.

These definitions devised by botanists, are not satisfactory thermodynamically because water should be considered to move down an energy gradient, losing energy as it does so. The term water potential (Ψ - Greek capital letter psi) is now used, and solutions of compounds in water are said to exert an osmotic potential. As the potential of pure water is defined as being zero, and dissolved substances cause it to be reduced, solutions have osmotic potentials, Ψs (or $\Psi\pi$ - Greek small letter pi) which are negative in value. Water is said to move from a region of high potential (having a less negative value) to one that is lower (having a more negative value). Both osmotic pressure and osmotic potential are used as terms in tissue culture literature and are equivalent except that they are opposite in sign (i.e. an OP of +6 bar equals a Ψs of -6 bar). Thermodynamically, the cell's turgor pressure is defined as a positive pressure potential (Ψp). The water potential of a cell ($\Psi cell$) is then equal to the sum of its osmotic and pressure potentials plus the force holding water in microcapillaries or bound to the cell wall matrix (Ψm):

$\Psi\ cell = \Psi s + \Psi p + \Psi m$

Modern statements of the older suction pressure concept are therefore that the difference in water potential (i.e. the direction of water movement) between a cell and solution outside is given by:

$\Delta\Psi = (\Psi s_{inside\ cell} – \Psi s_{outside\ cell}) – \Psi p$ or

$\Delta\Psi = \Psi\ cell – \Psi\pi_{outside}$

and when $\Delta\Psi = 0$ (at equilibrium)

$\Psi\pi_{outside} = \Psi\ cell$

The force of water movement between two cells, `

'A' and 'B', of different water potential, is given by:

$\Delta\Psi = \Psi$ cellA – ΨcellB

the direction of movement being towards the more negative water potential.

The osmotic potential (pressure) of solutions is determined by their molar concentration and by temperature. The water potential of a plant tissue culture medium (Ψtcm) is equivalent to the osmotic potential of the dissolved compounds (Ψs). There is no pressure potential but, if they are added, substances such as agar and Gelrite contribute a matric potential (Ψm):

Ψtcm = Ψs + Ψm

Osmotic pressure and water potential are measured in standard pressure units thus:

1 bar = 0.987 atm
= 10^6 dynes cm^{-2}
= 10^5 Pa (0.1 MPa = 1 bar).

Whereas molarity is defined as number of gram moles of a substance in one litre of a solution (i.e. one litre of solution requires less than one litre of solvent), molality is the number of gram moles of solute per kilogram of solvent, and thus, unlike osmotic potential (osmolality, measured in pressure units) is independent of temperature. It is therefore more convenient to give measurements of osmotic pressure in osmolality units. The osmole (Osm) is defined as:

The unit of the osmolality of a solution exerting an osmotic pressure equal to that of an ideal non-dissociating substance which has a concentration of one mole of solute per kilogram of solvent.

The osmolality of a very dilute solution of a substance which does not dissociate into ions, will be the same as its molality (i.e. g moles per kilogram of solvent). The osmolality of a weak solution of a salt, or salts, which has completely dissociated into ions, will equal that of the total molality of the ions.

The osmotic potential of dilute solutions approximates to Van't Hoff's equation: Ψ = -cRT; where

c = concentration of solutes in mol/litre;
R = the gas constant and
T = temperature in °K

From the above equation, at 0°C one litre of a solution containing 1 mole of an undissociated compound, or 1 mole of ions, could be expected to have an osmolality of 1 Osm/kg, and an osmotic (water) potential of:

Ψ= -1 (mole) x 0.082054 (atm /mole/°C)
x 273.16 (°K) = - 22.414 atm

Thus, although in practice the Van't Hoff equation must be corrected by the osmotic coefficient, Φ:

$\Psi = -\Phi$ cRT (Lang, 1967),

it is possible to give approximate figures for converting osmolality into osmotic potential pressure units. These are shown in Table 4.3. This table can also be used to estimate the osmotic potential of non-dissociating molecules such as sugars or mannitol. Thus at 25°C, 30 g/l sucrose (molecular wt. 342.3) should exert an osmotic potential of:

$$-2.4789 \text{ x } \frac{30}{342.3} = -0.217 \text{ MPa}$$

The observed potential is -0.223 Mpa (Table 4.4).

A definition. The osmotic properties of solutions can be difficult to describe without confusion. In this book, the addition of solutes to a solvent (which makes the osmotic or water potential more negative, but makes the osmolality of the solution increase to a larger positive value), has been said to <u>reduce</u>

Table 4.3 Factors by which osmolality (Osm/kg) should be multiplied to estimate an equivalent osmotic potential in pressure units.

For conversion to	Multiply Osm/kg by the factor shown for an equivalent osmotic potential in pressure units[1]				
	15 ºC	20 ºC	0 ºC	25 ºC	30 ºC
Atm	-22.414	-23.645	-24.055	-24.465	-24.875
Bar	-22.711	-23.958	-24.374	-24.789	-25.205
Dyne/cm^2	-22.711 x 10^6	-23.958 x 10^6	-24.374 x 10^6	-24.789 x 10^6	-25.205 x 10^6
Mpa	-2.2711	-2.3958	-2.4374	-2.4789	-2.5205

* Divide pressure units by the figures shown to find approximate osmolality,

$$\text{e.g.} -223 \text{ kPa} = \frac{-223}{1000} \text{ x } \frac{1}{-2.4789} = 0.090 \text{ Osm/kg.}$$

Table 4.4 The osmolality and osmotic potential of sucrose solutions at different concentrations.

Sucrose concentration		Osmolality	Osmotic potential at
(% , w/v)	(mM)	(Osm/kg)	25 ºC (MPa)
0.5	14.61	0.015	-0.037
1.0	29.21	0.030	-0.074
1.5	43.82	0.045	-0.112
2.0	58.43	0.060	-0.149
2.5	73.04	0.075	-0.186
3.0	87.64	0.090	-0.223
4.0	116.86	0.121	-0.300
5.0	175.28	0.186	-0.461
6.0	233.71	0.253	-0.627
8.0	292.14	0.324	-0.803
10.0	350.57	0.396	-0.982

(decrease) the osmotic potential of solutions. MS medium containing 3% sucrose (osmolality, ca. 186 mOsm/kg; ca. -461 kPa at 25°C) is thus described as having a lower water potential than the medium of White (1954) supplemented with 2% sucrose (osmolality, ca. 78 mOsm/kg; ca. −193 kPa at 25°C).

4.2. THE OSMOTIC POTENTIAL OF TISSUE CULTURE MEDIA

4.2.1. The total osmotic potential of solutes

The approximate total osmotic potential of a medium due to dissolved substances, can be estimated from: $\Psi s = \Psi s_{macronutrients} + \Psi s_{sugars}$

When 3% w/v sucrose is added to Murashige and Skoog (1962) medium, the osmolality of a filter sterilised preparation rises from 0.096 to 0.186 Osm/kg and at 25°C, the osmotic potential of the medium decreases from -0.237 to -0.460 MPa. Sugars are thus responsible for much of the osmotic potential of normal plant culture media. Even without any inversion to monosaccharides, the addition of 3% w/v sucrose is responsible for over four fifths of the total osmotic potential of White (1954) medium, 60% of that of Schenk and Hildebrandt (1972), and for just under one half that of MS.

The contribution of the gelling agent. The water potential of media solidified with gels is more negative than that of a liquid medium, due to their matric potential, but this component is probably relatively small (Amador and Stewart, 1987). In the following sections, the matric potential of semi-solid media containing ca. 6 g/l agar has been assumed to be -0.01 MPa at 25°C, but as adding extra agar to media helps to prevent hyperhydricity, it is possible that this is an underestimate.

The contribution of nutrient salts. Inorganic salts dissociate into ions when they are dissolved in water, so that the water potential of their solutions (especially weak solutions) does not depend on the molality (or molarity) of undissociated compounds, but on the molality (or molarity) of their ions. Thus a solution of KCl with a molality of 0.1, will have a theoretical osmolality of 0.2, because in solution it dissociates into 0.1 mole K^+ and 0.1 mole Cl^-. Osmolality of a solution of mixed salts is dependent on the total molality of ions in solution.

Dissociation may not be complete, especially when several different compounds are dissolved together as in plant culture media, which is a further reason why calculated predictions of water potential may be imprecise. In practice, osmotic potentials should be determined by actual measurement with an osmometer. Clearly though, osmotic potential of a culture medium is related to the concentration of solutes, particularly that of the macronutrients and sugar.

Of the inorganic salts in nutrient media, the macronutrients contribute most to the final osmotic (water) potential because of their greater concentration. The osmolality of these relatively dilute solutions is very similar to the total osmolarity of the constituent ions at 0°C, and can therefore be estimated from the total molarity of the macronutrient ions. Thus based on its macronutrient composition, a liquid Murashige and Skoog (1962) medium (without sugar) with a total macronutrient ion concentration of 95.75 mM, will have an osmolality of ca. 0.0958 osmoles (Osm) per kilogram of water solvent (95.8 mOsm/kg), at 25°C, an osmotic potential of ca. -0.237 MPa (237 kPa). Estimates of osmolality

derived in this way agree closely to actual measurements of osmolality or osmolarity for named media given in the papers of Yoshida *et al.* (1973), Kavi Kishor and Reddy (1986) and Lazzeri *et al.* (1988) (see Table 4.5).

The contribution of sugars. The osmolality and osmotic potential of sucrose solutions can be read from Table 4.6. Those of mannitol and sorbitol solutions of equivalent molarities will be approximately comparable. At concentrations up to 3% w/v, the osmolality of sucrose is close to molarity. It will be seen that the osmotic potential (in MPa) of sucrose solutions at 25°C can be roughly estimated by multiplying the % weight/volume concentration by -0.075: that of the monosaccharides fructose, glucose, mannitol and sorbitol (which have a molecular weight approximately 0.52 times that of sucrose), by multiplying by -0.14.

If any of the sucrose in a medium becomes hydrolysed into monosaccharides, the osmotic potential of the combined sugar components (sucrose + glucose + fructose), (Ψs_{sugars}), will be lower (more negative) than would be estimated from Table 4.5. The effect can be seen in Table 4.6. From this data it seems that 40-50% of the sucrose added to MS medium by Lazzeri *et al.* (1988) was broken down into monosaccharides during autoclaving. Hydrolysis of sucrose by plant-derived invertase enzymes will also have a similar effect on osmotic potential. In some suspension cultures, all sucrose remaining in the medium is inverted within 24 h. A fully inverted sucrose solution would have almost double the negative potential of the original solution, but as the appearance of glucose and fructose by enzymatic hydrolysis usually occurs concurrently with the uptake of sugars by the tissues, it will tend to have a stabilizing effect on osmotic potential during the passage of a culture. Reported measurements of the osmolality of MS medium containing 3% sucrose, after autoclaving, are:

230 mOsm/kg (0.65% Phytagar; Lazzeri *et al.,* 1988)

240 ± 20 mOsm/kg (0.6-0.925% agar; Scherer *et al.,* 1988)

230 ± 50 mOsm/kg (0.2-0.4% Gelrite; Scherer *et al.,* 1988)

4.2.2. Decreasing osmotic potential with other osmotica

By adding soluble substances in place of some of the sugar in a medium, it can be shown that sugars not only act as a carbohydrate source, but also as osmoregulants. Osmotica employed for the deliberate modification of osmotic potential, should be largely lacking other biological effects. Those most frequently selected are the sugar alcohols mannitol and sorbitol. It is assumed that plants that do not have a native pathway for sugar alcohol biosynthesis are also deficient in pathways to assimilate them. Sugar alcohols, though, are usually translocated, and may be metabolised and utilized to various degrees (Steinitz, 1999; for mannitol Lipavska and Vreugedenhil, 1996 Tian and Russell, 1999; for sorbitol Pua *et al.,* 1984). Polyethylene glycol may be more helpful as an inert nonpenetrating osmolyte although it may contain toxic contaminants (Chazen *et al.,* 1995). Mannitol can easily penetrate cell walls, but the plasmalemma is considered to be relatively impermeable to it (Rains, 1989), whereas high-molecular-weight polyethylene glycol 4000 is too large to penetrate cell walls (Carpita *et al.,* 1979; Rains, 1989). Thus, a nonpenetrating osmolyte cannot penetrate into the plant cells, but inhibits water uptake. Sodium sulphate and sodium chloride have also been used in some experiments.

Table 4.5. Predicted osmolality and the osmolality actually observed after autoclaving (data from Lazzeri *et al.,* 1988).

	Predicted osmolality (no sucrose hydrolysis)				Oberved total osmolality (mOsm/kg)
	Salts (mOsm/kg)	Agar (mOsm/kg)	Sucrose (mOsm/kg)	Total (mOsm/kg)	
MS + agar	96	4	-	100	-
MS + agar + 0.5% sucrose	96	4	15	115	115
MS + agar + 1.0% sucrose	96	4	30	130	140
MS + agar + 2.0% sucrose	96	4	60	160	184†
MS + agar + 4.0% sucrose	96	4	121	221	276

† 161 mOsm/kg by Brown *et al.* (1989)

Table 4.6. The osmolality and osmotic potential of sucrose solutions of different concentrations.

Sucrose Concentration		**Osmolality**	**Osmotic potential at 25°C**
(% w/v)	**mM**	**Osm/Kg**	**MPa**
0.5	14.61	0.015	-0.037
1.0	29.21	0.030	-0.074
1.5	43.82	0.045	-0.112
2.0	58.43	0.060	-0.149
2.5	73.04	0.075	-0.186
3.0	87.64	0.090	-0.223
4.0	116.86	0.121	-0.300
6.0	175.28	0.186	-0.461
8.0	233.71	0.253	-0.627
10.0	292.14	0.324	-0.803
12.0	350.57	0.396	-0.982

4.3. EFFECTS AND USES OF OSMOLYTES IN TISSUE CULTURE MEDIA

4.3.1. Protoplast isolation and culture

The osmotic potential of a plant cell is counter-balanced by the pressure potential exerted by the cell wall. To safely remove the cell wall during protoplast isolation without damaging the plasma membrane, it has been found necessary to plasmolyse cells before wall-degrading enzymes are used. This is done by placing the cells in a solution of lower water potential than that of the cell.

Glucose, sucrose and especially mannitol and sorbitol, are usually added to protoplast isolation media for this purpose, either singly or in combination, at a total concentration of 0.35-0.7 M. These addenda are then retained in the subsequent protoplast culture medium, their concentration being progressively reduced as cell colonies start to grow. Tobacco cell suspensions take up only a very small amount of mannitol from solution (Thompson and Thorpe, 1981) and its effect as an osmotic agent appears to be exerted outside the cell (Thorpe, 1982). When protoplasts were isolated from *Pseudotsuga* and *Pinus* suspensions, which required a high concentration of inositol to induce embryogenesis , it was found to be essential to add 60 g/l (0.33 M) myo-inositol (plus 30 g/l sucrose, 20 g/l glucose and 10 g/l sorbitol) to the isolation and culture media (Gupta *et al.,* 1988). Mannitol and further amounts of sorbitol could not serve as substitutes.

4.3.2. Osmotic effects on growth

Solutions of different concentrations partly exert their effect on growth and morphogenesis by their nutritional value, and partly through their varying osmotic potential. Lapeña *et al* (1988) estimated that three quarters of the sucrose necessary to promote the optimum rate of direct adventitious shoot formation from *Digitalis obscura* hypocotyls, was required to supply energy, while the surplus regulated morphogenesis osmotically.

How osmotic potential influences cellular processes is still far from clear. Cells maintained in an environment with low (highly negative) osmotic potential, lose water and in consequence the water potential of the cell decreases. This brings about changes in metabolism and cells accumulate high levels of proline (Rabe, 1990). The activity of the main respiratory pathway of cells (the cytochrome pathway) is reduced in conditions of osmotic stress, in favour of an alternative oxidase system (De Klerk-Kiebert and Van der Plas, 1985). The increase of the concentration of osmolytes, may also result in high levels of the plant hormone abscisic acid, both *extra vitrum* and *in vitro* (recent reviews Zhu, 2002; Riera *et al.,* 2005).

Equilibrium between the water potential of the medium and that of *Echinopsis* callus, only occurred when the callus was dead. Normally the water potential of the medium was greater so that water flowed into the callus (Kirkham and Holder, 1981). Clearly this situation could not occur in media which were too concentrated, and Cleland (1977) proposed that a critical water potential needs to be established within a cell before cell expansion and cell division, can occur. The osmotic concentration of culture media could therefore be expected to influence the rate of cell division or the success of morphogenesis of the cells or tissues they support. Both the inorganic and organic components will be

contributory. The cells of many plants which are natives of sea-shores or deserts (e.g. many cacti) characteristically have a low water potential (Ψcell) and in consequence may need to be cultured in media of relatively low (highly negative) osmotic potentials (Lassocinski, 1985). Sucrose concentrations of 4.5-6% have sometimes been found to be beneficial for such plants (Sachar and Iyer, 1959; Johnson and Emino, 1979; Mauseth, 1979; Lassocinski, 1985).

Above normal sucrose concentrations can often be beneficial in media for anther culture [e.g 13% sucrose in Gamborg *et al.* (1968) B5 medium - Chuong and Beversdorf, 1985], and for the culture of immature embryos [e.g. 10% sucose in MS medium - Stafford and Davies (1979); 12.5% in Phillips and Collins (1979) L2. medium - Phillips *et al.* (1982)].

If the osmotic potential of the medium does indeed influence the growth of tissue cultures, one might expect the sucrose concentration, which is optimal for growth, to vary from one medium to another, more sucrose being required in dilute media than in more concentrated ones. Evans *et al.* (1976) found this was so with cultures of soybean tissue. Maximum rates of callus growth were obtained in media containing either:

1. 50-75% of MS basal salts + 3-4% sucrose, or,
2. 75-100% of MS basal salts + 2% sucrose.

Similarly Yoshida *et al.* (1973) obtained equally good growth rates of *Nicotiana glutinosa* callus with nutrient media in which

1. the salts exerted -0.274 MPa and sucrose -0.223 MPa, or
2. the salts exerted -0.365 MPa and sucrose -0.091 MPa.

It was essential to add 60 g/l sucrose (*i.e.* $\Psi s_{sucrose}$= -0.461 MPa at 25°C) to the 'MEDIUM' salts of de Fossard *et al.* (1974) (Ψ = -0.135 MPa) to obtain germination and seedling growth from immature zygotic embryos of tomato. However, if the 'HIGH' salts (Ψs_{medium} = -0.260 MPa) were used, 60 g/l sucrose gave only slightly better growth than 2.1 g/l ($\Psi s_{sucrose}$ = -0.156 MPa) (Neal and Topoleski, 1983).

A detailed examination of osmotic effects of culture media on callus cultures was conducted by Kimball *et al.* (1975). Various organic substances were added to a modified Miller (1961) medium (which included 2% sucrose), to decrease the osmotic potential (Ψs) from -0.290 MPa. Surprisingly, the greatest callus growth was said to occur at -1.290 to -1.490 MPa (unusually low potentials which normally inhibit growth — see below) in the presence of mannitol or sorbitol, and between -1.090 to -1.290 MPa when extra sucrose or glucose were added. On the standard medium, many cells of the callus were irregularly shaped; as the osmotic potential of the solution was decreased there were fewer irregularities and at about Ψs = -1.090 MPa all the cells were spherical. The percentage dry matter of cultures also increased as Ψs was decreased.

Doley and Leyton (1970) found that decreasing the water (osmotic) potential of half White (1963) medium by -0.100 or -0.200 MPa through adding more sucrose (and/or polyethylene glycol), caused the rate of callus growth from the cut ends of *Fraxinus* stem sections to be lower than on a standard medium. At the reduced water potential, callus had suberised surfaces and grew through the activity of a vascular cambium. It also contained more lignified xylem and sclereids. At each potential there was an optimal IAA concentration for xylem differentiation.

When the concentration of sucrose in a high salt medium such as MS is increased above 4-5 per cent, there begins to be a progressive inhibition of cell growth in many types of culture. This appears to be an osmotic effect because addition of other osmotically-active substances (such as mannitol and polyethyleneglycol) to the medium causes a similar response (Maretzki *et al.,* 1972). Usually, high concentrations of sucrose are not toxic, at least not in the short term, and cell growth resumes when tissues or organs are transferred to media containing normal levels of sugar. Increase of Ψs is one method of extending the shelf life of cultures. Pech and Romani (1979) found that the addition of 0.4 M mannitol to MS medium (modified organics), was able to prevent the rapid cell lysis and death which occurred when 2,4-D was withdrawn from pear suspension cultures.

Decreasing the osmotic potential (usually by adding mannitol) together with lowering of the temperature has been used to reduce the growth rate for preservation of valuable genotypes *in vitro*. This has been reported, among others, for potato (Gopal *et al.,* 2002; Harding *et al.,* 1997), *Dioscorea alata* (Borges *et al.,* 2003) and enset (Negash *et al.,* 2001)

4.3.3. Tissue water content

The water content of cultured tissues decreases as the level of sucrose in the medium is increased. Isolated embryos of barley were grown by Dunwell (1981) on MS medium in sucrose concentrations up to 12 per cent. Dry weight increased as the sucrose concentration was raised to 6 or 9 per cent and shoot length of some varieties was also greater than on 3 per cent sucrose. Water content of the developing

plantlets was inversely proportional to the sucrose level.

The dry weight of *Lilium auratum* bulbs and roots on MS medium increased as the sucrose concentration was augmented to 90 g/l but decreased dramatically with 150 g/l because growth was inhibited. The fresh weight/dry weight ratio of both kinds of tissue once again declined progressively as sucrose was added to the medium (0 - 150 g/l). In a medium containing 30 mg/l sucrose, the number and fresh weight of bulblets increased as MS salt strength was raised from one eighth to two times its normal concentration. An interaction between salt and sucrose concentrations was demonstrated in that optimum dry weight of bulbs could be obtained in either single strength MS + 120 g/l sucrose (osmolality, without sucrose inversion = ca. 492 mOsm/kg; Ψs = ca. -1.22 MPa), or double strength MS + 60 g/l sucrose (osmolality, without sucrose inversion = ca. 378 mOsm/kg; Ψs = ca. -0.94 MPa) (Takayama and Misawa, 1979).

4.3.4. Morphogenesis

The osmotic effect of sucrose in culture solutions was well demonstrated by a series of experiments on tobacco callus by Brown *et al.,* (1979): rates of callus growth and shoot regeneration which were optimal on culture media containing 3 per cent sucrose, could be maintained when the sugar was replaced partially by osmotically equivalent levels of mannitol. The optimal (medium plus sucrose) here was between -0.4 and -0.6 MPa, and increasing sucrose levels above 3 per cent brought a progressive decrease in shoot regeneration. Similar results were obtained by Barg and Umiel (1977), but when they kept the osmotic potential of the culture solution roughly constant by additions of mannitol, the sucrose concentrations optimal for tobacco callus growth or morphogenesis were not the same (Fig. 4.2)

Brown and Thorpe (1980) subsequently found that callus of *Nicotiana* capable of forming shoots, had a water potential (Ψ) of -0.8 MPa, while non-shoot-forming callus had a Ψ of -0.4 MPa. The two relationships were:

Shoot forming callus

$\Psi cell = \Psi s + \Psi p + \Psi m$

$-0.8 = -1 + 0.4 + 0$ MPa

Non-shoot-forming callus

$\Psi cell = \Psi s + \Psi p + \Psi m$

$-0.4 = -0 + 0.3 + 0$ MPa

Correct water potential. An optimum rate of growth and adventitious shoot formation of wheat callus occurred on MS medium containing 2% sucrose. Only a small number of shoots were produced on the medium supplemented with 1% sucrose, but if mannitol was added so that the total Ψcell = Ψs was the same as when 2% sucrose was present, the formation of adventitious shoots was stimulated (Galiba and Erdei, 1986). Very similar results were obtained by Lapeña *et al.* (1988). A small number of shoot buds were produced from *Digitalis obscura* hypocotyls on MS medium containing 1% sucrose: more than twice as many if the medium contained 2% sucrose (total Ψs given as -0.336 MPa), or 1% sucrose plus mannitol to again give a total Ψs equal to -0.336 MPa.

Water potential can modify commitment. Morphogenesis can also be regulated by altering the water potential of media. Shepard and Totten (1977) found that very small (ca. 1-2 mm) calluses formed from potato mesophyll protoplasts were unable to survive in 1 or 2% sucrose, and the base of larger (5-10 mm) ones turned brown, while the upper portions turned green but formed roots and no shoots. The calli became fully green only on 0.2-0.5% sucrose. At these levels shoots were formed in the presence of 0.2-0.3 M mannitol. When the level of mannitol was reduced to 0.05 M, the proportion of calli differentiating shoots fell from 61% to 2%. The possibility of an osmotic affect was suggested because equimolar concentrations of myo-inositol were just as effective in promoting shoot regeneration.

Another way to modify morphogenesis is to increase the ionic concentration of the medium. Pith phloem callus of tobacco proliferates on Zapata *et al.* (1983) MY1 medium supplemented with 10^{-5} M IAA and 2.5 x 10^{-6} M kinetin, but forms shoots on Murashige *et al.* (1972) medium containing 10^{-5} M IAA and 10^{-5} M kinetin. These two media contain very similar macronutrients (total ionic concentrations, respectively 96 and 101mM), yet adding 0.5-1.0% sodium sulphate (additional osmolality 89-130 mOsm/kg) decreased the shoot formation of callus grown on the shoot-forming medium, but increased it on the medium which previously only supported callus proliferation (Pua *et al.,* 1985a). Callus cultured in the presence of sodium sulphate retained its shoot-producing capacity over a long period, although the effect was not permanent (Pua *et al.,* 1985b; Chandler *et al.,* 1987). In these experiments shoot formation was also enhanced by sodium chloride and mannitol.

Increasing the level of sucrose from 1 to 3 per cent in MS medium containing 0.3 mg/l IAA,

induced tobacco callus to form shoots, while further increasing it to 6 per cent resulted in root differentiation (Rawal and Mehta, 1982; Mehta, 1982). The formation of adventitious shoots from *Nicotiana tabacum* pith callus is inhibited on a medium with MS salts if 10-15% sucrose is added. Preferential zones of cell division and meristemoids produced in 3% sucrose then become disorganised into parenchymatous tissue (Hammersley-Straw and Thorpe, 1988).

It should be noted that auxin which has a very important influence on the growth and morphogenesis of cultured plant cells, causes their osmotic potential to be altered (Van Overbeek, 1942; Hackett, 1952; Ketellapper, 1953). When tobacco callus is grown on a medium which promotes shoot regeneration, the cells have a greater osmotic pressure (or more negative water potential) than callus grown on a non-inductive medium (Brown and Thorpe, 1980; Brown, 1982).

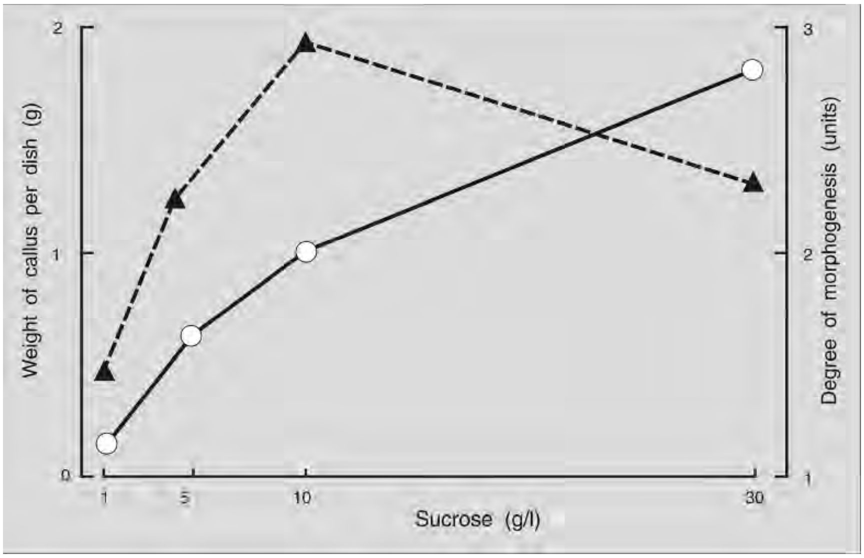

Fig. 4.2 The effect of sucrose concentration on the growth and morphogenesis of tobacco callus. [Drawn from data for four lines of tobacco callus in Barg and Umiel, 1977]. Callus growth = solid line. Morphogenesis = line of dashes. Scale was, 1 = No differentiation, 2 = Dark green callus with meristemoids, 3 = leafy shoots

Apogamous buds on ferns. Whittier and Steeves (1960) found a very clear effect of glucose concentration on the formation of apogamous buds on prothalli of the fern *Pteridium*. (Apogamous buds give rise to the leafy and spore-producing generation of the plant which has the haploid genetic constitution of the prothallus). Bud formation was greatest between 2-3% glucose (optimum 2.5%). Results which confirm this observation were obtained by Menon and Lal (1972) in the moss *Physcomitrium pyriforme*. Here apogamous sporophytes were formed most freely in low sucrose concentrations (0.5-2%) and low light conditions (50-100 lux), and were not produced at all when prothalli were cultured in 6% sucrose or high light (5000-6000 lux). Whittier and Steeves (loc. cit.) noted that they could not obtain the same rate of apogamous bud production by using 0.25% glucose plus mannitol or polyethyleneglycol; on the other hand, adding these osmotica to 2.5% glucose (so that the osmotic potential of the solution was equivalent to one with 8% sucrose) did reduce bud formation. It therefore appeared that the stimulatory effect of glucose on morphogenesis was mainly due to its action as a respiratory substrate, but that inhibition might be caused by an excessively depressed osmotic potential.

Differentiation of floral buds. Pieces of cold-stored chicory root were found by Margara and Rancillac (1966) to require more sucrose (up to 68 g/l; 199 mM) to form floral shoots than to produce vegetative shoots (as little as 17 g/l; 50 mM). Tran Thanh Van and co-workers (Tran Thanh Van and Trinh, 1978) have similarly shown that the specific formation of vegetative buds, flower buds, callus or roots by thin cell layers excised from tobacco stems, could be controlled by selecting appropriate concentrations of sugars and of auxin and cytokinin growth regulators.

Root formation and root growth. Media of small osmotic potential are usually employed for the

induction and growth of roots on micropropagated shoots. High salt levels are frequently inhibitory to root initiation. Where such levels have been used for Stage II of shoot cultures, it is common to select a low salts medium (e.g. ¼ or ½ MS), when detached shoots are required to be rooted at Stage III. By testing four concentrations of MS salts (quarter, half, three quarters and full strength) against four levels of sucrose (1, 2, 3 and 4%), Harris and Stevenson (1979) found that correct salt concentration (½ or ¼ MS) was more important than sucrose concentration for root induction on grapevine cuttings *in vitro*. The benefit of low salt levels for root initiation may be due more to the need for a low nitrogen level, than for an increased osmotic potential. Dunstan (1982) showed that microcuttings of several tree-fruit rootstocks rooted best on MS salts, but that in media of these concentrations, the amount of added sugar was not critical, although it was essential for there to be some present. For the best rooting of *Castanea*, it was important to place shoots in Lloyd and McCown (1981) WPM medium containing 4% sucrose (Serres, 1988).

There are reports that an excessive sugar concentration can inhibit root formation. Green cotyledons of *Sinapis alba* and *Raphanus sativus* were found by Lovell *et al.* (1972) to form roots in 2% sucrose in the dark, but not in light of 5500 lux luminous intensity. In the light, rooting did occur if the explants were kept in water, or (to a lesser extent) if they were treated with DCMU (a chemical inhibitor of photosynthesis) before culture in 2% sucrose. The authors of this paper suggested that sugars were produced within the plant tissues during photosynthesis, which, added to the sucrose absorbed from the medium, provided too great a total sugar concentration for rooting. Rahman and Blake (1988) reached the same conclusion in experiments on *Artocarpus heterophyllus*. When shoots of this plant were kept on a rooting medium in the dark, the number and weight of roots formed on shoots, increased with the inclusion of up to 80 g/l sucrose. The optimum sucrose concentration was 40 g/l if the shoots were grown in the light.

Root formation on avocado cuttings in 0.3 MS salts [plus Linsmaier and Skoog (1965) vitamins] was satisfactory with 1.5, 3 or 6% sucrose, and only reduced when 9% sucrose was added (Pliego-Alfaro, 1988).

Although 4% (and occasionally 8%) sucrose has been used in media for isolated root culture, 2% has been used in the great majority of cases (Butcher and Street, 1964). In an investigation into the effects of sucrose concentration on the growth of tomato roots, Street and McGregor (1952) found that although sucrose concentrations of between 1.5 and 2.5% caused the same rate of increase of root fresh weight, 1.5% sucrose was optimal. It produced the best rate of growth of the main root axis, and the greatest number and total length of lateral roots.

Somatic embryogenesis. The osmotic potential of a medium can influence whether somatic embryogenesis can occur and can regulate the proper development of embryos. As will be shown below, a low osmotic potential is often favourable, but this is not always the case. For instance immature cotyledons of *Glycine max* produced somatic embryos on Phillips and Collins (1979) L2 medium containing less than 2% sucrose, but not if the concentration of sugar was increased above this level (Lippmann and Lippmann, 1984).

Placing tissues in solutions with high osmotic potential will cause cells to become plasmolysed, leading to the breaking of cytoplasmic interconnections between adjacent cells (plasmodesmata). Wetherell (1984) has suggested that when cells and cell groups of higher plants are isolated by this process, they become enabled to develop independently, and express their totipotency. He pointed out that the isolation of cells of lower plants induces regeneration, and plasmolysis has long been known to initiate regeneration in multicellular algae, the leaves of mosses, fern prothallia and the gemmae of liverworts (Narayanaswami and LaRue, 1955; Miller, 1968). Carrot cell cultures pre-plasmolysed for 45 min in 0.5-1.0 M sucrose or 1.0 M sorbitol gave rise to many more somatic embryos when incubated in Wetherell (1969) medium with 0.5 mg/l 2,4-D than if they had not been pre-treated in this way. Moreover embryo formation was more closely synchronized. Ikeda-Iwai *et al.* (2003) found that in *Arabidopsis* a 6-12 hour treatment with 0.7 M sucrose, sorbitol or mannitol resulted in somatic embryogenesis.

Callus derived from hypocotyls of *Albizia richardiana*, produced the greatest numbers of adventitious shoots on B5 medium containing 4% sucrose, but somatic embryos grew most readily when 2% sucrose was added. At least 1% sucrose was necessary for any kind of morphogenesis to take place (Tomar and Gupta, 1988). A similar result was obtained by Ćulafić *et al.* (1987) with callus from axillary buds of *Rumex acetosella*: adventitious shoots were produced on a medium containing MS

salts and 2% sucrose (Ψs = ca. -0.39 MPa at 25°C), but embryogenesis occurred when the sucrose concentration was increased to 6% ($\Psi s_{sucrose}$ = -0.46 MPa, Ψs = ca. -0.70 MPa at 25°C) or if the medium was supplemented with 2% sucrose plus 21.3 g/l mannitol or sorbitol (which together have the same osmolality as 6% sucrose).

A low (highly negative) osmotic potential helps to induce somatic embryogenesis in some other plants. Adding 10-30 g/l sorbitol to Kumar *et al.* (1988) L-6 medium (total macronutient ions 64.26 mM; 20 g/l sucrose), caused there to be a high level of embryogenesis in *Vigna aconitifolia* suspensions and the capacity for embryogenesis to be retained in long-term cultures. The formation of somatic embryos in ovary callus of *Fuchsia hybrida* was accelerated by adding 5% sucrose to B5 medium (Dabin and Beguin, 1987), and the induction of embryogenic callus of *Euphorbia longan* required the culture of young leaflets on B5 medium with 6% sucrose (Litz, 1988).

There are exceptions, particularly with regard to embryo growth. The proportion of *Ipomoea batatas* somatic embryos forming shoots was greatest when a medium containing MS inorganics contained 1.6%, rather than 3% sucrose (Chée *et al.,* 1990). Protocorm proliferation of orchids is most rapid when tissue is cultured in high concentrations of sucrose, but for plantlet growth, the level of sucrose must be reduced (Homès and Vanseveran-Van Espen, 1973).

The induction of embryogenic callus from immature seed embryos of *Zea mays* was best on MS medium with 12% sucrose (Lu *et al.,* 1982), and Ho and Vasil (1983) used 6-10% sucrose in MS medium to promote the formation of pro-embryoids from young leaves of *Saccharum officinarum*. However, in the experiments of Ahloowalia and Maretski (1983), somatic embryo formation from callus of this plant was best on MS medium with 3% sucrose, but growth of the embryos into complete plantlets required that the embryos should be cultured first on MS medium with 6% sucrose and then on MS with 3% sucrose.

Polyethylene glycol 4000 (PEG 4000) improves root and shoot emergence without limiting embryo histodifferentiation in soybean somatic embryos (Walker and Parrott, 2001). Likewise in spruce, it was reported that polyethylene glycol might improve the quality of somatic embryos by promoting normal differentiation of the embryonic shoot and root (e.g. Stasolla *et al.,* 2003). Non-penetrating osmotica like polyethylene glycol cannot enter plant cells, but restrict water uptake and provide a simulated drought stress during embryo development. A combination of ABA and an osmoticum prevents precocious germination in white spruce (Attree *et al.,* 1991) and allows embryo development to proceed. Advantageous effects of polyethylene glycol and ABA have been reported in a number of species (*Hevea brasiliensis*, Linossier *et al.,* 1997; *Picia abies*, Bozhkov and Von Arnold, 1998; white spruce, Stasolla *et al.,* 2003, *Corydalis yanhusuo*, Sagare *et al.,* 2000; and *Panax ginseng*, Langhansová *et al.,* 2004).

When cultured on Sears and Deckard (1982) medium, embryogenesis in callus initiated from immature embryos of 'Chinese Spring' and some other varieties of wheat was incomplete, because shoot apices germinated and grew before embryos had properly formed. More typical somatic embryos could be obtained by adding 40 mM sodium or potassium chloride to the medium. The salts had to be removed to allow plantlets to develop normally (Galiba and Yamada, 1988). Transferring somatic embryos to a medium of lower (more negative) water potential is often necessary to ensure their further growth and/or germination. High sucrose levels are often required in media for the culture of zygotic embryos if they are isolated when immature. The use of 50-120 g/l sucrose in media is then reported, the higher concentrations usually being added to very weak salt mixtures. Embryos which are more fully developed when excised, grow satisfactorily in a medium with 10-30 g/l sugar.

Storage organ formation. At high concentration, sucrose promotes the formation of tubers, bulbs and corms (e.g. Xu *et a*l., 1998; Vreugdenhil *et al*., 1998; Ziv 2005, Gerrits and de Klerk 1992). This promotion might be mediated by ABA since osmotic stress induces ABA synthesis (Riera *et al*., 2005) and ABA promotes bulb (Kim *et al*., 1994) and tuber (Xu *et al*., 1998) formation. The situation is, however, more complex. Suttle and Hultstrand (1994) did not find a reduction of tuber formation in potato by adding fluridone, an ABA-synthesis inhibitor, and Xu *et al.* (1998) did not observe an increased ABA-level at high sucrose concentration. So in potato, the effect of sucrose is not likely to be mediated by ABA. Exogenous ABA does not promote *Gladiolus* corm formation (Dantu and Bhojwani, 1995) but bulb formation of lily was completely inhibited by fluridone and restored by simultaneous addition of ABA (Kim *et al*., 1994). To establish the effect of osmoticum directly, experiments have been carried out with addition of

mannitol instead of sucrose. Mannitol did not promote corm production of *Gladiolus* (De Bruyn and Ferreira, 1992) or bulb production in onion (Kahane and Rancillac, 1996), but data for lily suggested that, although it had a toxic effect, in this plant mannitol did stimulate bulb formation (Gerrits and De Klerk, 1992).

Anther culture. The use of high concentrations of sucrose is commonly reported in papers on anther culture where the addition of 5-20% sucrose to the culture medium is found to assist the development of somatic embryos from pollen microspores. This appears to be due to an osmotic regulation of morphogenesis (Sunderland and Dunwell, 1977), for once embryoid development has commenced, such high levels of sucrose are no longer required, or may be inhibitory. A high concentration of mannitol has been used for pretreatment before the culture of barley anthers (Roberts-Oehlschlager and Dunwell 1990) and pollen (Wei *et al*., 1986); tobacco anthers (Imamura and Harada 1980) and pollen (Imamura *et al.,* 1982); and before wheat microspore culture (Hu *et al.,* 1995). A high concentration of mannitol has also been used to induce osmotic stress in microspore derived embryos of *Brassica napus* (Huang *et al.,* 1991) and before anther culture of *Brassica campestris* (Hamaoka *et al.,* 1991). Isolated microspores of *Brassica napus* cultured on a high concentration of mannitol and at a low concentration of sucrose (0.08–0.1%) yield no embryos whereas on high polyethyleneglycol 4000 the embryo yield is comparable to that of the sucrose control (Ikeda-Iwai, 2003). These results demonstrate that in microspore embryogenesis of *Brassica napus* the level of metabolizable carbohydrate required for microspore embryo induction and formation may be very low and that an appropriate osmoticum (polyethylene glycol 4000 or sucrose) is required.

The temporary presence of high sucrose concentrations is said to prevent the proliferation of callus from diploid cells of the anther that would otherwise swamp the growth of the pollen-derived embryoids. The concentration of macronutrient ions generally used in anther culture media is not especially low. In a sample of reports it was found to be 68.7 mM (George *et al*., 1987), and so the total osmotic potential, Ψ_s, (salts plus sucrose) of many anther culture media is in the range -0.55 to -1.15 MPa.

4.3.5. Relative humidity.

The vapour pressure of water is reduced by dissolving substances in it. This means that the relative humidity of the air within closed culture vessels is dependent on the water potential of the medium according to the equation:

$$\Psi = \frac{1000RT}{W_0} \ln_e\left(\frac{p}{p_0}\right) \cdot \left(p - c\frac{dp}{dc}\right)$$

(after Glasstone, 1947)

where: Ψ, R and T are as in the Van't Hoff equation, W_0 is the molecular weight of water, c is the concentration of the solution in moles per litre, and p_0 and p are respectively the vapour pressures of water and the solution.

If the change in vapour pressure dependent on the density of the solution, $p - c$ (dp/dc), is treated as unity (legitimate perhaps for very dilute solutions, or when the equation is expressed in molality, rather than molarity), it is possible to estimate relative humidity ($100 \times p/p_0$) above tissue culture media of known water potentials, from:

$$\Psi = \frac{1000RT}{18.016} \ln_e\left(\frac{p}{p_0}\right), \text{ (Lang, 1967)}$$

The relative humidity above most plant tissue cultures in closed vessels is thus calculated to be in the range 99.25-99.75% (Table 4.7), the osmolality of some typical media being

- White (1963), liquid, 2% sucrose, 106 mOsm/kg
- MS, agar, 3% sucrose, 230 mOsm/kg
- MS, agar, 6% sucrose, 359 mOsm/kg
- MS, agar, 12% sucrose (unusual), 659 mOsm/kg

(in these cases, 50% hydrolysis of sucrose into monosaccharides is assumed to have taken place during autoclaving)

Relative humidity can be reduced below the levels indicated above by, for example, covering vessels with gas-permeable closures while using non-gelatinous support systems (see Section 6.3.1).

Table 4.7 The relative humidity within culture vessels which would be expected to result from the use of plant culture media of various osmolalities or water potentials

Relative humidity in vessel (%)	Osmolality (mOsm/kg)	Water potential (kPa) at temperature:			
		15 ºC	20 ºC	25 ºC	30 ºC
98	1121	-2686	-2733	-2780	-2826
99	558	-1337	-1360	-1383	-1406
99.5	278	-667	-678	-690	-701
99.75	139	-333	-339	-344	-350

5. pH OF TISSUE CULTURE MEDIA

The relative acidity or alkalinity of a solution is assessed by its pH. This is a measure of the hydrogen ion concentration; the greater the concentration of H^+ ions (actually H_3O^+ ions), the more acid the solution. As pH is defined as the negative logarithm of hydrogen ion concentration, acid solutions have low pH values (0-7) and alkaline solutions, high values (7-14). Solutions of pH 4 (the concentration of H^+ is 10^{-4} mol.l^{-1}) are therefore more acid than those of pH 5 (where the concentration of H^+ is 10^{-5} mol.l^{-1}); solutions of pH 9 are more alkaline than those of pH 8. Pure water, without any dissolved gases such as CO_2, has a neutral pH of 7. To judge the effect of medium pH, it is essential to discriminate between the various sites where the pH might have an effect: (1) in the explant, (2) in the medium and (3) at the interface between explant and medium.

The pH of a culture medium must be such that it does not disrupt the plant tissue. Within the acceptable limits the pH also:

- governs whether salts will remain in a soluble form;
- influences the uptake of medium ingredients and plant growth regulator additives;
- has an effect on chemical reactions (especially those catalysed by enzymes); and
- affects the gelling efficiency of agar.

This means that the effective range of pH for media is restricted. As will be explained, medium pH is altered during culture, but as a rule of thumb, the initial pH is set at 5.5 – 6.0. In culture media, detrimental effects of an adverse pH are generally related to ion availability and nutrient uptake rather than cell damage.

5.1. THE pH OF MEDIA

5.1.1. Buffering

The components of common tissue culture media have only little buffering capacity. Vacin and Went (1949) investigated the effect on pH of each compound in their medium. The chemicals which seemed to be most instrumental in changing pH were $FeSO_4.7H_2O$ and $Ca(NO_3)_2.4H_2O$. Replacing the former with ferric tartrate at a weight which maintained the original molar concentration of iron, and substituting $Ca_3(PO_4)_2$ and KNO_3 for $Ca(NO_3)_2.4H_2O$, they found that the solution was more effectively buffered. While amino acids also showed promise as buffering agents, KH_2PO_4 was ineffective unless it was at high concentration. In some early experiments attempts were made to stabilise pH by incorporating a mixture of KH_2PO_4 and K_2HPO_4 into a medium (see Kordan, 1959, for example), but Street and Henshaw (1966) found that significant buffering was only achieved by soluble phosphates at levels inhibitory to plant growth. For this reason Sheat *et al.* (1959) proposed the buffering of plant root culture media with sparingly-soluble calcium phosphates, but unfortunately if these compounds are autoclaved with other medium constituents, they absorb micronutrients which then become unavailable.

A buffer is a compound which can poise the pH level at a selected level: effective buffers should maintain the pH with little change as culture proceeds. As noted before, plant tissue culture media are normally poorly buffered. However, pH is stabilised to a certain extent when tissues are cultured in media containing both nitrate and ammonium ions. Agar and Gelrite gelling agents may have a slight buffering capacity (Scherer, 1988).

Organic acids: Many organic acids can act as buffers in plant culture media. By stabilizing pH at ca. pH 5.5, they can facilitate the uptake of NH_4^+ when this is the only source of nitrogen, and by their own metabolism, assist the conversion of NH_4^+ into amino acids. There can be improvement to growth from adding organic acids to media containing both NH_4^+ and NO_3^-, but this is not always the case. Norstog and Smith (1963) noted that 0.75 mM malic

acid was an effective buffer and appeared to enhance the effect of the glutamine and alanine which they added to their medium. Vyskot and Bezdek (1984) found that the buffering capacity of MS medium was increased by adding either 1.25 mM sodium citrate or 1.97 mM citric acid plus 6.07 mM dibasic sodium phosphate. Citric acid and some other organic acids have been noted to enhance the growth of *Citrus* callus when added to the medium (presumably that of Murashige and Tucker, 1969) (Erner and Reuveni, 1981). For the propagation of various cacti from axillary buds, Vyskot and Jara (1984) added sodium citrate to MS medium to increase its buffering capacity.

Recognized biological buffers: Unlike organic acids, conventional buffers are not metabolised by the plant, but can poise pH levels very effectively. Compounds which have been used in plant culture media for critical purposes such as protoplast isolation and culture, and culture of cells at very low inoculation densities, include:

- TRIS, Tris(hydroxymethyl)aminomethane;
- Tricine, N-tris(hydroxymethyl)methylglycine;
- MES, 2-(N-morpholino)ethanesulphonic acid;
- HEPES, 4-(2-hydroxyethyl)-1-piperazine(2-ethanesulphonic acid); and
- CAPS, 3-cyclohexylamino-1-propanesulphonic acid.

Such compounds may have biological effects which are unrelated to their buffering capacity. Depending on the plant species, they have been known to kill protoplasts or greatly increase the rate of cell division and/or plant regeneration (Conrad *et al.,* 1981). The 'biological' buffers MES and HEPES have been developed for biological research (Good *et al.,* 1966).

MES: MES is one of the few highly effective and commercially available buffers with significant buffering capacity in the pH range 5-6 to which plant culture media are usually adjusted and has only a low capacity to complex with micronutrients. It is not toxic to most plants, although there are some which are sensitive. Ramage and Williams (2002) report that shoot regeneration from tobacco leaf discs was not affected by MES when increasing the concentration up to 100 mM. De Klerk et al (2007), though, observed a decrease of rooting from apple stem slices with increasing MES concentration (see below; Fig. 4.4). This effect of MES was not understood. It only occurred during the first days of the rooting process and was not observed during the outgrowth phase after the meristems had been formed.

During the culture of thin cell layers of *Nicotiana*, Tiburcio *et al.* (1989) found that the pH of LS medium could be kept close to 5.8 for 28 days by adding 50 mM MES, whereas without the buffer pH gradually decreased to 5.25. Regulating pH with MES alters the type of morphogenesis which occur in this (and other) tissues (see below).

Parfitt *et al.* (1988) found 10 mM MES to be an effective buffer in four different media used for tobacco, carrot and tomato callus cultures, and peach and carnation shoot cultures, although stabilizing pH did not result in superior growth. The tobacco, peach and carnation cultures were damaged by 50 mM MES. Tris was toxic at all concentrations tested, although Klein and Manos (1960) had found that the addition of only 0.5 mM Tris effectively increased the fresh weight of callus which could be grown on White (1954) medium when iron was chelated with EDTA.

MES has also been used successfully to buffer many cultures initiated from single protoplasts (Müller *et al.,* 1983) and its inclusion in the culture medium can be essential for the survival of individual cells and their division to form callus colonies (e.g. those of *Datura innoxia* - Koop *et al.,* 1983). MES was found to be somewhat toxic to single protoplasts of *Brassica napus*, but 'Polybuffer 74' (PB-74, a mixture of polyaminosulphonates), allowed excellent microcolony growth in the pH range 5.5-7.0 (Spangenberg *et al.,* 1986). Used at $^1/_{100}$ of the commercially available solution, it has a buffering capacity of a 1.3 mM buffer at its pK value (Koop *et al.,* 1983).

Banana homogenate is widely used in orchid micropropagation media. Ernst (1974) noted that it appeared to buffer the medium in which slipper orchid seedlings were being grown.

5.1.2. The uptake of ions and molecules

The pH of the medium has an effect on the availability of many minerals (Scholten and Pierik, 1998). In general, the uptake of negatively charged ions (anions) is favoured at acid pH, while that of cations (positively charged) is best when the pH is increased. As mentioned before, the relative uptake of nutrient cations and anions will alter the pH of the medium. The release of hydroxyl ions from the plant in exchange for nitrate ions results in media becoming more alkaline; when ammonium ions are

Table 4.8 The uptake of ions and its consequences in plant culture media

	Uptake anion (e.g. NH_4^+)	**Uptake cation** (e.g. NO_3^-)
Rate	Best in alkaline or weakly acid solutions	Best in relatively acid solutions
Consequence	Protons (H^+) extruded by plant Medium becomes more ACID	Hydroxyl (OH^-) ions extruded by plant Medium becomes more ALKALINE

taken up in exchange for protons, media become more acid (Table 4.8).

Nitrate and ammonium ions: The uptake of ammonium and nitrate ions is markedly affected by pH. Excised plant roots can be grown with NH_4^+ as the sole source of nitrogen providing the pH is maintained within the range 6.8 to 7.2 and iron is available in a chelated form. At pH levels below 6.4 the roots grow slowly on ammonium alone and have an abnormal appearance (Sheat et al.,1959). This accords with experiments on intact plants where ammonium as the only source of nitrogen is found to be poorly taken up at low pH. It was most effectively utilised in *Asparagus* in a medium buffered at pH 5.5. At low pH (ca. 4 or less) the ability of the roots of most plants to take up ions of any kind may be impaired, there may be a loss of soluble cell constituents and growth of both the main axis and that of laterals is depressed (Asher, 1978).

Nitrate on the other hand, is not readily absorbed by plant cells at neutral pH or above (Martin and Rose, 1976). Growth of tumour callus of *Rumex acetosa* on a nitrate-containing medium was greater at pH 3.5 than pH 5.0 (Nickell and Burkholder, 1950), while Chevre *et al.* (1983) reported that axillary bud multiplication in shoot cultures of *Castanea* was most satisfactory when the pH of MS was reduced to 4 and the Ca^{2+} and Mg^{2+} concentrations were doubled.

pH Stabilization: One of the chief advantages of having both NO_3^- and NH_4^+ ions in the medium is that uptake of one provides a better pH environment for the uptake of the other. The pH of the medium is thereby stabilized. Uptake of nitrate ions by plant cells leads to a drift towards an alkaline pH, while NH_4^+ uptake results in a more rapid shift towards acidity (Street, 1969; Behrend and Mateles, 1975; Hyndman *et al.,* 1982). For each equivalent of ammonium incorporated into organic matter, about 0.8-1 H^+ (proton) equivalents are released into the external medium; for each equivalent of nitrate assimilated, 1-1.2 proton equivalents are removed from the medium (Fuggi *et al.,* 1981). Raven (1986) calculated that there should be no change of pH resulting from NO_3^- or NH_4^+ uptake, when the ratio of the two is 2 to 1.

The pH shifts caused by uptake of nitrate and ammonium during culture (see above) can lead to a situation of nitrogen deficiency if either is used as the sole nitrogen source without the addition of a buffer (see later) (Hyndman *et al.,* 1982). The uptake of NH_4^+, when this is the sole source of nitrogen is only efficient when a buffer or an organic acid is also present in the medium. In media containing both NO_3^- and NH_4^+ with an initial pH of 5-6, preferential uptake of NH_4^+ causes the pH to drop during the early growth of the culture. This results in increased NO_3^- utilisation (Martin and Rose, 1976) and a gradual pH rise. The final pH of the medium depends on the relative proportions of NO_3^- and NH_4^+ which are provided (Gamborg *et al.,* 1968). After 7 days of root culture, White (1943a) medium (containing only nitrate), adjusted to pH 4.8-4.9, had a pH of 5.8-6.0 (Street *et al.,* 1951, 1952), but Sheat *et al.* (1959) could stabilize the medium at pH 5.8 by having one fiftieth of the total amount of nitrogen as ammonium ion, the rest as nitrate. Changes in the pH of a medium do however vary from one kind of plant to another. Ramage and Williams (2002) observed a decrease in pH when tobacco leaf discs were cultured with only NH_4^+ nitrogen whereas no such decrease was observed on medium with both NH_4^+ and NO_3^-. No organogenesis occurred when the medium with only NH_4^+ was unbuffered but the inclusion of MES resulted in the formation of meristems (but no shoots).

Media differing in total nitrogen levels (but all having the same ratio of nitrate to ammonium as MS medium), had a final pH of ca. 4.5 after being used for Stage III root initiation on rose shoots, whereas those containing ammonium alone had a final pH of ca. 4.1 (Hyndman *et al.,* 1982). A similar observation with MS medium itself was made by Delfel and Smith (1980). No matter what the starting value in the range 4.5-8.0, the final pH after culture of *Cephalotaxus* callus was always 4.2. The medium of De Jong *et al.* (1974) always had a pH of 4.8-5.0 after *Begonia* buds had been cultured, whatever the

starting pH in the range 4.0-6.5. This is not to say that the initial pH was unimportant, because there was an optimum for growth and development (see later) (Berghoef and Bruinsma, 1979).

Other Compounds: The availability and uptake of other inorganic ions and organic molecules is also affected by pH. As explained above, the uptake of phosphate is most efficient from acid solutions. Petunia cells took up phosphate most rapidly at pH 4 and its uptake declined as the pH was raised (Chin and Miller, 1982). Vacin and Went (1949) noted the formation of iron phosphate complexes in their medium by pH changes. Insoluble iron phosphates can also be formed in MS medium at pH 6.2 or above unless the proportion of EDTA to iron is increased (Dalton *et al.,* 1983).

The uptake of Cl^- ions into barley roots is favoured by low pH, but Jacobson *et al.* (1971) noticed that it was only notably less at high pH in solutions strongly stirred by high aeration. They therefore suggested that H^+ ions secreted from plant roots as a result of the uptake of anions, can maintain a zone of reduced pH in the Nernst layer, a stationary film of water immediately adjacent to cultured plant tissue (Nernst, 1904). In plant tissue culture, uptake of ions and molecules may therefore more liable to be affected by adverse pH in agitated liquid media, than in media solidified by agar.

Lysine uptake into tobacco cells was found by Harrington and Henke (1981) to be stimulated by low pH. The effect of pH on uptake is especially relevant for auxins (e.g., Edwards and Goldsmith 1980). Depending on the pH and their pKa, auxins are either present as an undissociated molecule or as an anion. For influx, the undissociated auxin molecule may pass through the membrane by diffusion whereas the anion is taken up by a carrier (Delbarre *et al.,* 1996; Morris 2000). Dissociation dependens upon the pH. In the apoplast, the pH is low, ca. 5. When taken up, the auxin enters the cytoplasm with pH 7. At this pH, most auxin is present as anion and cannot diffuse out. Efflux of the anion is brought about by an efflux carrier system. Thus, the net uptake into cells of plant growth regulators which are weak lipophilic acids (such as IAA, NAA, 2,4-D and abscisic acid) will be greater, the more acid the medium and the greater the difference between its pH and that of the cell cytoplasm (Rubery, 1980). Shvetsov and Gamburg (1981) did in fact find that the rate of 2,4-D uptake into cultured corn cells increased as the pH of the medium fell from 5.5 to 4. Increased uptake of auxin at low pH was also found in apple microcuttings, both for IBA (Harbage *et al.,* 1998) and IAA (De Klerk *et al.,* 2007). As previously mentioned, auxins can themselves modify intra- and extra-cellular pH. Adding 2,4-D to a medium increased the uptake of nicotine into culture of *Acer pseudoplatanus* cells (Kurkdjian *et al.,* 1982).

5.1.3. Choosing the pH of culture media: Starting pH

Many plant cells and tissues *in vitro*, will tolerate pH in the range of about 4.0-7.2; those inoculated

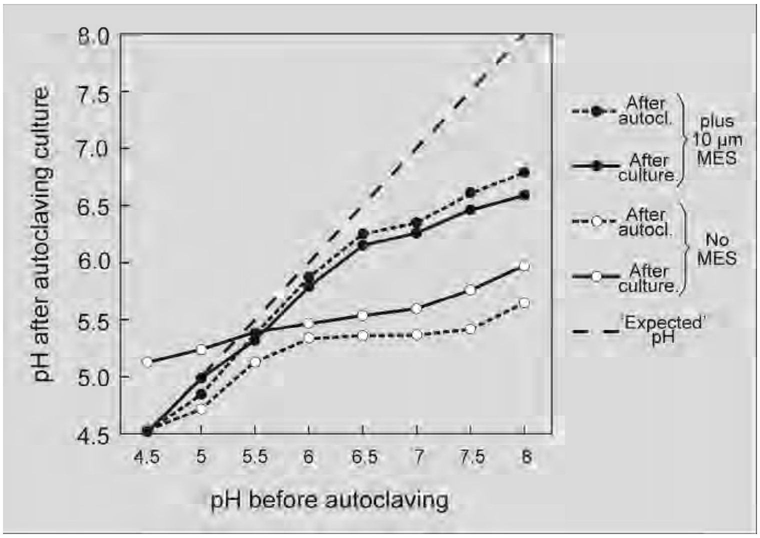

Fig. 4.3 Development of pH during tissue culture. The pH was set before autoclaving as is usually done, and measured directly after autoclaving and after 5 days of culture with 1-mm stem-slices cut from apple microshoots. The medium was in Petri dishes with BBL agar and modified MS medium. Per Petri dish, 30 ml of medium was added and 30 slices were cultured. (Data from de Klerk *et al.,* 2007).

into media adjusted to pH 2.5-3.0 or 8.0 will probably die (Butenko *et al.,* 1984). Best results are usually obtained in slightly acid conditions. In a random sample of papers on micropropagation, the average initial pH adopted for several different media was found to be 5.6 (mode 5.7) but adjustments to as low as 3.5 and as high as 7.1 had been made.

The pH for most plant cultures is thus lower than that which is optimum for hydroponic cultures, where intact plants with their roots in aerated solution usually grow most rapidly when the pH of the solution is in the range 6.0-7.3 (De Capite, 1948; Sholto Douglas, 1976; Cooper, 1979).

Suspension cultured cells of *Ipomoea*, grew satisfactorily in Rose and Martin (1975) P2 medium adjusted initially to pH 5.6 or pH 6.3, but the yield of dry cells was less at two extremes (pH 4.8 and pH 7.1). Martin and Rose (1976) supposed that a low yield of cells from a culture started at pH 7.1 was due to the inability of the culture to utilise NO_3^-, but the cause of a reduction in growth at pH 4.8 was less obvious. It might have resulted from the plant having to expend energy to maintain an appropriate physiological pH internally.

Kartha (1981) found that pH 5.6-5.8 supported the growth of most meristem tips in culture and that cassava meristems did not grow for a prolonged period on a medium adjusted to pH 4.8. The optimum pH (before autoclaving) for the growth of carnation shoot tips on Linsmaier and Skoog (1965) medium, was 5.5-6.5. When the medium was supplemented with 4 mg/l adenine sulphate and 2 g/l casein hydrolysate, the optimum pH was 5, and on media adjusted to 6.0 and 6.5, there was significantly less growth (Davis *et al.,* 1977). Shoot proliferation in *Camellia sasanqua* shoot cultures was best when the pH of a medium with MS salts was adjusted to 5-5.5. Only in these flasks was the capacity of juvenile explants to produce more shoots than adult ones really pronounced (Torres and Carlisi, 1986).

Norstog and Smith (1963) suggested that the pH of the medium used for the culture of isolated zygotic embryos, should not be greater than 5.2.

5.1.4. pH adjustment

Because there is then no need to take special aseptic precautions, and it is impractical to adjust pH once medium has been dispensed into small lots, the pH of a medium is usually adjusted with acid or alkali before autoclaving. According to Krieg and Gerhardt (1981), agar is partially hydrolysed if autoclaved at pH 6 or less and will not solidify so effectively when cooled. They recommend that agar media for bacteriological purposes should be sterilised at a pH greater than 6 and, if necessary, should be adjusted to acid conditions with sterile acid after autoclaving, when they have cooled to 45-50°C. The degree of hydrolysis in plant culture media autoclaved at pH 5.6-5.7, is presumably small.

The effect of autoclaving: Autoclaving changes the pH of media (Fig. 4.3). In media without sugars, the change is usually small unless the phosphate concentration is low, when more significant fluctuations occur. Media autoclaved with sucrose generally have a slightly lower pH than those autoclaved without it, but if maltose, glucose, or fructose have been added instead of sucrose, the post-autoclave pH is significantly reduced (Owen *et al.,* 1991).

The pH of liquid media containing MS salts [e.g. Linsmaier and Skoog (1965) or Skirvin and Chu (1979) media] containing 3-3.4% sucrose, has been found to fall during autoclaving from an adjusted level of 5.7, to pH 5.17 (Singha, 1982), to pH 5.5 (Owen *et al.,* 1991), or to pH 4.6 (Skirvin *et al.,* 1986). The drop in pH may vary according to the pH to which the medium was initially adjusted. In the experiments of Skirvin *et al.* (loc. cit.), the pH of a medium adjusted to 5.0, fell to 4.2; one adjusted to 6.4, fell to 5.1; that set at pH 8.5, fell to 8.1.

Most agars cause the pH of media to increase immediately they are dissolved. Knudson (1946) noticed that the pH of his medium shifted from 4.6-4.7 to 5.4-5.5 once agar had been added and dissolved; and Singha (1982) discovered that the unadjusted pH of MS medium rose from pH 4 to pH 5.2, depending on the amount of agar added. However if a medium containing agar was adjusted to pH 5.7, and then autoclaved, the medium became more acid than if agar had not been added, the fall in pH being generally in proportion to the amount of agar present.

The effect of storage. The pH of autoclaved plant media tends to fall if they are stored. Vacin and Went (1949) noted that autoclaving just accelerated a drop in the pH of Knudson (1946) C medium, as solutions left to stand showed similar changes. Sterilisation by filtration (see below) was not a satisfactory alternative, as it too effected pH changes. The compounds particularly responsible were thought to be unchelated ferrous sulphate (when the pH had initially been set between 3 and 6), and calcium nitrate (when the original pH was 6 to 9). Complex iron phosphates, unavailable to plants, were produced from the ferrous sulphate, but if iron was added as

ferric citrate or ferric tartrate (chelates), no significant pH changes resulted from autoclaving, and plants showed no iron-deficiency symptoms.

Skirvin *et al.* (1986) found that both with and without agar, autoclaved MS medium tended to become more acid after 6 weeks storage, for example:

Time	MS medium	
	Liquid	With 0.6% Difco Bacto Agar
Starting pH	5.7	5.7
After autoclaving	4.6	4.6
6 weeks later	4.1	4.4

To minimise a change in the pH of stored media, it is suggested that they are kept in the dark: Owen *et al.,* (1991) reported that the pH of MS containing 0.1M sucrose or 0.8% Phytagar, remained relatively stable after autoclaving if kept in the dark at 4°C, but fell by up to 0.8 units if stored in the light at 25°C. De Klerk *et al.* (2007) using BBL agar, also observed a shift of pH, but this was negligible when 10 mM MES was added (Fig. 4.3).

Hydrolysis: Some organic components of culture media are liable to be hydrolysed by autoclaving in acid media. The degree of hydrolysis of different brands of agar may be one factor influencing the incidence of hyperhydricity in plant cultures. Agar media may not solidify satisfactorily when the initial pH has been adjusted to 4-4.5. The reduction in pH which occurs in most media during autoclaving may also cause unsatisfactory gelling of agar which has been added in low concentrations. Part of the sucrose added to plant culture media adjusted to pH 5.5 is also hydrolysed during autoclaving: the proportion degraded increases if the pH of the medium is much less than this. Hydrolysis of sucrose is, however, not necessarily detrimental.

Activated charcoal: The presence of activated charcoal can alter the pH of a medium. As in the production of activated charcoal it is, at one stage washed with HCl, the pH of a medium can be lowered by acid residues (Owen *et al.,* 1995; Wann *et al.,* 1997).

4.1.5. pH changes during culture

Due to the differential uptake of anions and cations into plant tissues, the pH of culture media does not usually stay constant, but changes as ions and compounds are absorbed by the plant. It is usual for media containing nitrate and ammonium ions to decline slowly in pH during a passage, after being adjusted initially to pH 5.4-5.7, Sometimes after a preliminary decrease, the pH may begin to rise and return to a value close to, or even well above that at which the culture was initiated. The pH of White (1954) medium, which contains only NO_3^- nitrogen, drifted from an initial 5.0 to 5.5 towards neutrality as callus was cultured on it (Klein and Manos, 1960).

With some cultures, the initial pH of the medium may have little effect. Cell suspension cultures of *Dioscorea deltoides* in the medium of Kaul and Staba (1968) [containing MS salts], adjusted to a pH either 3.5, 4.3, 5.8 or 6.3, all had a pH of 4.6-4.7 within 10 h of inoculation. There was a further fall to pH 4.0-4.2 in the next 2-3 days, but during the following 15-17 days the pH was 4.7-5.0, finally rising to pH 6.0-6.3 on about day 19 (Butenko *et al.,* 1984). Similarly, Skirvin *et al.* (1986) found that MS medium adjusted to pH 3.33, 5.11, 6.63, or 7.98 before autoclaving, and then used for the culture of *Cucumis melo* callus, had a pH after 48 h in the range 4.6-5.0. Visseur (1987) also reported that although the pH of his medium (similar macronutrients to MS but more Ca^{2+} and PO_4^{3-}) decreased if it was solidified with agar, but on a 2-phase medium, the final pH was 6.9 ± 0.4 irrespective of whether it was initially 4.8, 5.5 or 6.2.

Despite the above remarks, it should be noted that the nature of the pH drift which occurs in any one medium, differs widely, according to the species of plant grown upon it. The pH of the medium supporting shoot cultures of *Disanthus cercidifolius* changed from 5.5 to 6.5 over a 6 week period, necessitating frequent subculturing to prevent the onset of senescence, whereas in the medium in which shoots of the calcifuge *Lapageria rosea* were grown, the pH, initially set to 3.5-5.0, only changed to 3.8-4.1 (Howard and Marks, 1987). Note also that the reversion of media to a homeostatic pH, may be due to the presence of both NO_3^- and NH_4^+ ions (Dougall, 1980). Adjustment of media containing only one of these nitrogen sources to a range of pH levels, would be expected to result in a more variable set of final values.

As the pH of media deviate from the original titration level, simple unmonitored cultures may not provide the most favourable pH for different phases of growth and differentiation. In *Rosa* 'Paul's Scarlet' suspensions, the optimum pH for the cell division phase was 5.2-5.4: pH 5.5-6.0 was best for the cell expansion phase (Nesius and Fletcher, 1973). The maximum growth rate of *Daucus carota* habituated callus on White (1954) medium with iron as Fe-EDTA, occurred at pH 6.0 (Klein and Manos, 1960).

It has been suggested that acidification of media is partly due to the accumulation of carbon dioxide in tightly closed culture flasks (Leva *et al.,* 1984), but the decrease in pH associated with incubating anther cultures with 5% CO_2 was found by Johansson and Eriksson (1984) to be only ca. 0.1 units. Removal of CO_2 from an aerated cell suspension culture of *Poinsettia* resulted in an increase of about 0.2 pH-units (Preil, 1991).

Auxin plant growth regulators promote cell growth by inducing the efflux of H^+ ions through the cell wall. Hydrogen ion efflux from the cell is accompanied by potassium ion influx. When cultures are incubated in a medium containing an auxin, the medium will therefore become more acid while the pH of the cell sap will rise. The extent of these changes was found by Kurkdjian *et al.* (1982) to be proportional to auxin (2,4-D) concentration.

5.2. pH CONTROL WITHIN THE PLANT

The various compartments of cells have a different pH and this pH is maintained (Felle, 2001). In the symplasm, the pH of the cytoplasm is ca. 7 and of the vacuole ca. 5. The apoplasm has a pH of ca. 5. Plant cells typically generate an excess of acidic compounds during metabolism which have to be neutralised (Felle, 1998). One of the most important ways by which this is accomplished is for H^+, or K^+ to be pumped out of the cell, in exchange for anions (e.g. OH^-), thereby decreasing the extracellular pH. Plant cells also compensate for an excess of H^+ by the degradation of organic acids. Synthesis of organic acids, such as malate, from neutral precursors is used to increase H^+ concentration when the cytoplasmic pH rises, for instance if plants are grown in alkaline soils (Raven and Smith, 1976; Findenegg *et al.,* 1986). In intact plants, there is usually a downwards gradient from the low pH external to the cell, to higher pH levels in more mature parts, and this enables the upwards transport of non-electrolytic compounds such as sugars and amino acids (Böttger and Luthen, 1986).

Altering the pH of the external solution surrounding roots or cells can alter the pH of the cell (Smith and Raven, 1979). Because of necessary controls, the pH of the cytoplasm may be only slightly altered, that of vacuoles may show a more marked change. Changing the pH of the medium in which photo-autotrophic *Chenopodium rubrum* suspensions were cultured from 4.5 to 6.3, caused the pH of the cytoplasm to rise from 7.4 to 7.6 and that of the cell vacuoles to increase from 5.3 to 6.6. The increase in cytoplasmic pH caused there to be a marked diversion of carbon metabolism, away from sugar and starch, into the production of lipids, amino acids and proteins (Hüsemann *et al.,* 1990). The maintenance of the pH is also illustrated in an experiment with detached leaves of *Vicia faba* (Felle and Hanstein, 2002). When the leaves were placed in a 10 mM MES-TRIS buffer and transferred to buffer with another pH, changes in the pH of the apoplasm were small: with an initial buffer pH = 4.1 and transfer to buffer pH = 6.8, the apoplastic pH of substomatal cavities increased from 4.71 to 5.13 and in the reverse transfer decreased from 5.13 to 4.70. This indicates that the pH of the apoplasm is not strongly influenced by the medium but stays close to the 'natural' pH of ca. 5.0. No exact details are given but in this experiment the distance between the site of the pH measurements and the MES-TRIS solution in which the leaves had been placed was probably large. The symplasm has a much larger capacity to buffer (Felle, 2001) so that its pH will be even less influenced by the medium pH. Thus, within the explant the pH of both apoplasm and symplasm will be affected only little by the medium pH. The situation may be different at the interface between explant and medium. The influence of medium pH will extend towards inner tissues of the explant as the buffering capacity of the medium is increased (and thus overcomes buffering by the tissue). Inside the explant, the pH will also greatly influence movement

Table 4.9. The influence of the initial pH of Linsmaier Skoog (1965) medium on morphogenesis in thin cell layers of *Nicotiana* (data of Mutaftschiev *et al.,* 1987)

Initial pH of the medium	Mean number of organs per explant	Mean percentage of explants forming:			
		Callus	Roots	Vegetative buds	Flowers
3.8	4 ± 2	60 ± 10	40 ± 10	0	0
5.0	2 ± 1	90 ± 10	10 ± 10	0	0
6.1	20 ± 10	0	0	100	0
6.8	6 ± 2	0	0	20 ± 10	80 ± 10

through membranes, i.e. uptake in cells, as this often depends on the dissociation of compounds which is pH dependent.

Changing the pH of the medium can thus have a regulatory role on plant cultures which is similar to that of plant growth regulating chemicals, one of the actions of which is to modify intracellular pH and the quantity of free calcium ions. Auxins can modify cytoplasmic pH by triggering the release of H^+ from cells. In plant tissue culture these ions can acidify the medium (Kurkdjian *et al.,* 1982). Proton release is thought to be the first step in acid-triggered and turgor-triggered growth (Schubert and Matzke, 1985). It should be noted that pH changes themselves may act as a signal (Felle, 2001).

5.3. THE EFFECT OF pH ON CULTURES

5.3.1. Initiating cultures at low pH

Plants of the family Ericaceae which only grow well on acid soils (e.g. rhododendrons and blueberries), have been said to grow best on media such as Anderson (1975), Anderson (1978; 1980) and Lloyd and McCown (1981) WPM, when the pH is first set to ca. 4.5 (Anderson, 1975; Skirvin, 1981), but for highly calcifuge species such as *Magnolia soulangiana*, a starting pH of 3.5 can result in the highest rate of shoot proliferation in shoot cultures (Howard and Marks, 1987). Chevre *et al.* (1983) state that chestnut shoot cultures grew and proliferated best at pH 4 provided MS medium was modified by doubling the usual levels of calcium and magnesium.

De Jong *et al.* (1974), using a specially developed medium, found that a low pH value favoured the growth of floral organs. A similar result was seen by Berghoef and Bruinsma (1979c) with *Begonia* buds. Growth was greatest when the pH of the medium was initially adjusted to acid, 4.5-5.0 being optimal. At pH 4.0 the buds became glassy.

Before the discovery of effective chelating agents for plant cultures, root cultures were grown on media with a low pH. Tomato roots were, for instance, unable to grow on media similar to those of White (1943a), when the pH rose to 5.2 (Street *et al.,* 1951). Boll and Street (1951) were able to show that the depression of growth at high pH was due to the loss of Fe from the medium and that it could be overcome by adding a chelated form of iron (see chapter 3). Using FeEDTA, Torrey (1956) discovered that isolated pea roots [grown on a medium containing Bonner and Devirian (1939) A macronutrients, which do not contain NH_4^+], grew optimally at pH 6.0-6.4 but were clearly inhibited at pH 7.0 or greater; and Street (1969) reported that growth of tomato roots could be obtained between pH 4.0 and pH 7.2, if EDTA was present in the medium. Because agar does not gel properly when the initial pH of the medium is adjusted to 4, it is necessary to use liquid media for low pH cultures; or employ another gelling agent, or a mechanical support.

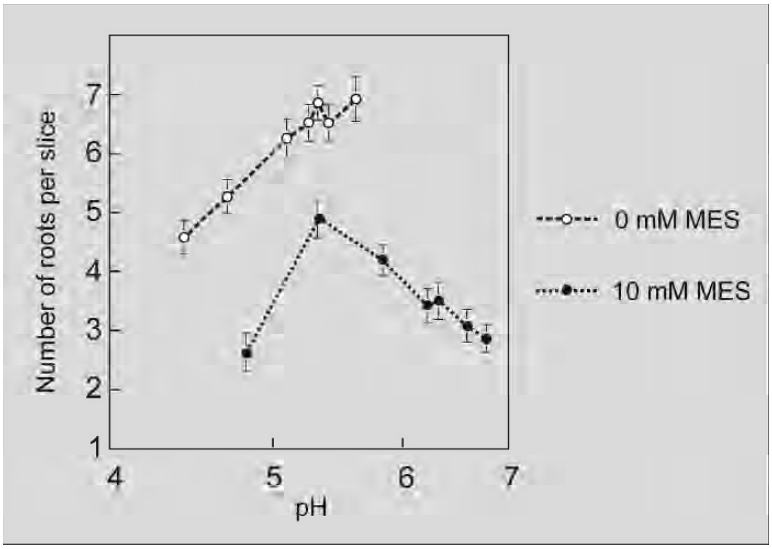

Fig. 4.4 Effect of medium pH on adventitious rooting from apple stem disks. The pH at the x-axis is the pH as measured at the start of culture after autoclaving (cf. Fig. 4.3). (from de Klerk *et al.,* 2007).

Some other cultures may also be beneficially started at low pH, which may indicate that the tissues have an initial requirement for NO_3^-. Embryogenesis of *Pelargonium* was induced more effectively if MS, or other media, were adjusted to pH 4.5-5.0 before autoclaving (rather than pH 5.5 and above) (Marsolais *et al.,* 1991).

5.3.2. Differentiation and Morphogenesis

Differentiation and morphogenesis are frequently found to be pH-dependent. Xylogenesis depends on the medium pH (Khan *et al.,* 1986). The growth of callus and the formation of adventitious organs from thin cell layers excised from superficial tissues of the inflorescence rachis of *Nicotiana*, depended on the initial pH of Linsmaier and Skoog (1965) medium containing 0.5 µM IBA and 3 µM kinetin (Table 4.9) (Mutaftschiev *et al.,* 1987). Pasqua *et al.* (2002) reported many quantitative effects of pH during regeneration from tobacco thin cell layers. The types of callus produced from the plumules of *Hevea* seedlings differed according to the pH of the medium devised by Chua (1966). Soft and spongy callus formed at acid (5.4) or alkaline (8.0) pH. A compact callus was obtained between pH 6.2 and 6.8.

5.3.3. Adventitious root formation

There are several reports in the literature which show that the pH of the medium can influence root formation of some plants *in vitro*. A slightly acid pH seems to be preferred by most species. Zatkyo and Molnar (1986), who showed a close correlation between the acidity of the medium (pH 7.0 to 3.0) and the rooting of *Vitis*, *Ribes nigrum* and *Aronia melancarpa* shoots, suggested that this was because acidity is necessary for auxin action.

Sharma *et al.* (1981) found it advantageous to reduce the pH of the medium to 4.5 to induce rooting of *Bougainvillea* shoots and a reduction of the pH of MS medium to 4.0 (accompanied by incubation in the dark) was required for reliable root formation of two *Santalum* species (Barlass *et al.,* 1980) and *Correa decumbens* and *Prostanthera striatifolia* (Williams *et al.,* 1984; 1985). Other Australian woody species rooted satisfactorily at pH 5.5 and pH 4 was inhibitory (Williams *et al.,* loc. cit.). Shoots from carnation meristem tips rooted more readily at pH 5.5 than pH 6.0 (Stone, 1963), and rooting of excised potato buds was best at pH 5.7, root formation being inhibited at pH 4.8 and at pH 6.2 or above (Mellor and Stace-Smith, 1969). Direct root formation on *Nautilocalyx* leaf segments was retarded if a modified MS medium containing IAA was adjusted initially to an acid pH (3.5 or 4.0) or a neutral pH (6.5). Good and rapid root formation occurred when the medium was adjusted to between pH 5.0 to 6.3 (Venverloo, 1976). De Klerk *et al* (2007), working with apple stem slices, found only a small effect of pH on rooting (Fig. 4.4): when the pH was set before autoclaving at 4.5 (after autoclaving the pH was 4.54), the number of roots was 4.5, and with the pH set at 8.0 (after autoclaving the pH had dropped to 5.65), the number of roots per slice increased to 7. In medium buffered with MES, the maximum number of roots was formed at pH 4.4 (measured after auto-claving). In these experiments, the dose-response curve for root number did not correspond with the effect of pH on IAA uptake. Such discrepancy between the effects of the pH on uptake and root number, was also reported by Harbage, Stimart and Auer (1998).

Direct formation of roots from *Lilium auratum* bulb scales occurred when MS medium was adjusted within the range 4-7 but was optimal at pH 6. The pH range for adventitious bulblet formation in this plant was from 4 to 8, but most bulblets were produced when the initial pH was between 5 and 7 (Takayama and Misawa, 1979).

Substrates which are to acid or too alkaline can adversely affect rooting *ex vitro*.

5.3.4. Embryogenesis

Smith and Krikorian (1989) discovered that pre-globular stage pro-embryos (PGSP) of carrot could be made to proliferate from tissues capable of direct embryo formation, with no auxin, on a medium containing 1-5 mM NH_4^+ (and no nitrate). Somatic embryos were formed when this tissue was moved to MS medium. The pH of the 'ammonium-nitrogen' medium fell from 5.5 to 4.0 in each subculture period, and it was later found (Smith and Krikorian, 1990a,b) that culture on a medium of low pH was essential for the maintenance of PGSP cultures. Sustained culture at a pH equal or greater than 5.7, with no auxin, allowed somatic embryo development. A similar observation to that of Smith and Krikorian had been made by (Stuart *et al.,* 1987). Although the pH of a suspension culture of alfalfa 'Regen-S' cells in a modified Schenk and Hildebrandt (1972) medium with 15 mM NH_4^+ was adjusted to 5.8, it quickly fell back to pH 4.4-5.0 in a few hours. The pH then gradually increased as somatic embryos were produced, until at day 14 it was 5.0. In certain suspension cultures, the pH was titrated daily to 5.5, but on each occasion soon returned to nearly the same pH as that in flasks which were untouched. Even so,

the pH-adjusted suspensions produced more embryos than the controls.

The ammonium ion has been found to be essential for embryogenesis. Is one of its functions to reduce the pH of the medium through rapid uptake and metabolism, thereby facilitating the uptake of nitrate, upon which embryogenesis is really dependent?

Embryogenesis from leaf explants of *Ostericum koreanum*, was found to depend strongly on pH (Cho *et al* 2003). As the explants were cultured continuously with NAA, it is possible that the observed relationship was caused by differential NAA uptake. This is also suggested by the slower rate of embryo development seen at low pH, because this would be expected where there is a high internal NAA concentration.

6. LIQUID MEDIA AND SUPPORT SYSTEMS

The nutritional requirements of plant cultures can be supplied by liquid media but growth in liquid medium may be retarded and development affected by oxygen deprivation and hyperhydration. The oxygen concentration of liquid media is often insufficient to meet the respiratory requirements of submerged cells and tissues. It can be increased either by raising the oxygen concentration of the medium or placing cells or tissues in direct contact with air. If the water potential of the medium is greater (less negative) than that of a cell, water flows into the cell and the vacuole becomes distended. Cells and tissues affected in this way are described as hyperhydric. Shoots often show physiological disturbances with symptoms that can be recognised visually (Chapter 13) (Debergh *et al.,* 1992; Gaspar *et al.,* 1987; Preece and Sutter, 1991; Ziv, 1991). The term hyperhydric is preferable to the previously used term 'vitrified' for reasons explained by Debergh *et al.* (1992). Water potential is determined by osmotic potential of the solutes in the medium and, in the case of a gelled medium, by the matric potential of the gel (Section 4.1) (Beruto *et al.,* 1995; Fujiwara and Kozai, 1995; Owens and Wozniak, 1991). Reductions in hyperhydricity can be achieved by increasing the concentrations of the solutes and the gel. Hyperhydricity may also be reduced through evaporation of water from tissues if they are placed in contact with air.

Contact of cultured tissues with air, to alleviate problems of hyperhydricity and hypoxia, can be achieved by the use of either porous or semi-solid (gelled) supports. The advantage of supports, as opposed to thin layers of liquid medium, is that tissues can be placed in a sufficient volume of medium to prevent depletion of nutrients and allow for the dispersion of any toxins that might be produced by the plant tissues. The relative advantages of liquid medium, solid supports or gelled media, varies with the type of material being cultured, the purpose of the culture and the scale of culture, as discussed below.

6.1. LIQUID MEDIA

Liquid medium, without supporting structures, is used for the culture of protoplasts, cells or root systems for the production of secondary metabolites, and the propagation of somatic embryos, meristematic nodules, microtubers and shoot clusters. In liquid medium, these cultures often give faster growth rates than on agar-solidifed medium. Cultures may be fully or only partially immersed in the medium.

Aeration of liquid medium in stationary Petri dishes is sometimes adequate for the culture of protoplasts and cells because of the shallow depth of the medium, but may still be suboptimal. Anthony *et al.* (1995) cultured protoplasts of cassava, in liquid medium in Petri dishes with an underlying layer of agarose in which glass rods were embedded vertically. Sustained protoplast division was observed in the cultures with glass rods but not in the controls without glass rods. The authors suggested that the glass rods extended the liquid meniscus, where the cell colonies were clustered thus causing gaseous exchange between the liquid and the atmosphere above to be facilitated.

Anthony *et al.* (1997) cultured protoplasts of *Passiflora* and *Petunia* in 30 ml glass bottles containing protoplast suspensions in 2 ml aliquots, either alone or in the presence of the oxygen carriers Erythrogen™ or oxygenated Perfluorodecalin. Cell division in each of the two species was stimulated by both oxygen carriers.

Laboratory-scale experimentation on immersed cultures of cells, tissues and organs, may be carried out in 125 ml or 250 ml Erlenmeyer flasks. Large-scale cultures are usually carried out in bioreactors with a capacity of 1 litre or more. The concentration of oxygen in the medium is raised by oxygen in the gas phase above and air bubbles inside the liquid. Increasing the oxygen concentration and circulation of the medium is facilitated in flasks by the use of gyratory shakers and in bioreactors by stirring and/or bubbling air through the medium (Chapter 1). The

use of bioreactors often involves the automated adjustment of the culture medium. The design of bioreactors for plant cells and organs was reviewed by Doran (1993) and the use of shake-flasks and bioreactors for the scale-up of embryogenic plant suspension cultures has been reviewed by Tautorus and Dunstan (1995). The importance of oxygen concentration in bioreactors can be illustrated by an investigation into the growth of hairy roots of *Atropa belladonna* (Yu and Doran, 1994). They found that no growth occurred at oxygen tensions of 50% air saturation but between 70% and 100% air saturation, total root length and the number of root tips increased exponentially. Hyperhydricity in liquid cultures may be avoided by adding to the medium osmoregulators, such as mannitol, maltose and sorbitol, and inhibitors of gibberellin biosynthesis including ancymidol and paclobutrazol (Ziv, 1989).

Plantlets and microtubers can be cultured by partial submersion in liquid medium. One method of aerating tissues is by the automated flooding and evacuation of tissues by liquid medium. This method has been used to produce microtubers of potato from single node cuttings (Teisson and Alvard, 1999). An alternative approach to aeration is to apply the liquid medium over the plant tissues as a nutrient mist. For example, Kurata *et al.* (1991) found that nodes of potato grew better in nutrient mist than on agar-based cultures.

6.2. SUPPORT BY SEMI-SOLID MATRICES

Gelled media provide semi-solid, supporting matrices that are widely used for protoplast, cell, tissue and organ culture. Agar, agarose, gellan gums and various other products have been used as gelling agents.

6.2.1. Agar

Agar is very widely employed for the preparation of semi-solid culture media. It has the advantages that have made it so widely used for the culture of bacteria, namely :-

- it forms gels with water that melt at approx. 100°C and solidify at approx. 45°C, and are thus stable at all feasible incubation temperatures;
- gels are not digested by plant enzymes;
- agar does not strongly react with media constituents.

To ensure adequate contact between tissue and medium, a lower concentration of agar is generally used for plant cultures than for the culture of bacteria. Plant media are not firmly gelled, but only rendered semi-solid. Depending on brand, concentrations of between 0.5-1.0% agar are generally used for this purpose. Agar is thought to be composed of a complex mixture of related polysaccharides built up from galactose. These range from an uncharged neutral polymer fraction, agarose, that has the capacity to form strong gels, to highly charged anionic polysaccharides, sometimes called agaropectins, which give agar its viscosity. Agar is extracted from species of *Gelidium* and other red algae, collected from the sea in several different countries. It varies in nature according to country of origin, the year of collection and the way in which it has been extracted and processed. The proportion of agarose to total polysaccharides can vary from 50 to 90% (Adrian and Assoumani, 1983). Agars contain small amounts of macro- and micro-elements; particularly calcium, sodium, potassium, and phosphate (Beruto *et al.,* 1995; Debergh, 1983; Scherer *et al.,* 1988), carbohydrates, traces of amino acids and vitamins (Day, 1942) that affect the osmotic and nutrient characteristics of a gel. They also contain phenolic substances (Scherer *et al.,* 1988) and less pure grades may contain long chain fatty acids, inhibitory to the growth of some bacteria.

As agar can be the most expensive component of plant media, there is interest in minimising its concentration. Concentrations of agar can be considered inadequate if they do not support explants or lead to hyperhydricity. Hyperhydricity decreases as the agar concentration is raised but there may be an accompanying reduction in the rate of growth. For example, Debergh *et al.* (1981) found that shoot cultures of *Cynara scolymus* were hyperhydric on medium containing 0.6% Difco 'Bacto' agar. No hyperhydricity occurred on medium containing 1.1% agar but shoot proliferation was reduced. Likewise, Hakkaart and Versluijs (1983) found that shoots of carnation were hyperhydric on medium containing 0.6% agar whereas growth was severely reduced on medium containing 1.2%. During studies to optimise the production of morphogenic callus from leaf discs of sugarbeet, Owens and Wozniak (1991) found large differences in the numbers of somatic embryos and shoots according to the gelling agent employed. They found that water availability, determined by gel matric potential, was the dominant factor involved. When they adjusted the concentrations of the gelling agents to give media of equal gel matric potential, somatic embryos and shoots were found in similar numbers on Bacto agar (0.7%), HGT agarose (0.46%), Phytagar (0.62%) and Gelrite (0.12%).

Various brands and grades of agar are available commercially. These differ in the amounts of impurities they contain and their gelling capabilities. The gelling capacity of Difco brands of agar increased with increasing purity i.e. 'Noble' > 'Purified' > 'Bacto' (Debergh, 1983). After impurities of agar were removed by sealing agar in a semi-permeable bags and washing in deionized water, the water potentials of gels of three brands were substantially lower than unwashed gels (Beruto *et al.,* 1995). A ten-fold difference in the regeneration rate of sugar cane was observed by Anders *et al.* (1988) on media gelled with the best and least effective of seven brands of agar. Scholten and Pierik (1998) investigated the different growth characteristics of seven different agar brands on the growth of axillary shoots, adventitious shoots and adventitious roots of rose, lily and cactus. They concluded that no single bioassay could identify 'good or bad' agars for a large group of plant species but Merk 1614, Daishin, MC29, and BD Purified gave the best results in most experiments. 'Daishin' showed no batch-to-batch variations. They found no relationship between price and quality of the brands of agar.

Agarose. Agarose is the high gel strength moiety of agar. It consists of β-D(1→3) galactopyranose and 3,6-anhydro-α-L(1 → 4) galactopyranose polymer chains of 20-160 monosaccharide units alternatively linked to form double helices. There are also several different agaro-pectin products available, which have been extracted from agar and treated to remove most of the residual sulphate side groupings (Shillito *et al.,* 1983). Because preparation involves additional processes, agarose is much more expensive than agar and its use is only warranted for valuable cultures, including protoplast and anther culture. Brands may differ widely in their suitability for these applications. Concentrations of 0.4-1.0% are used. Agarose derivatives are available which melt and gel at temperatures below 30°C, making them especially suitable for testing media ingredients that are heat-labile, or for embedding protoplasts. Low melting-point agarose is prepared by introducing hydroxyethyl groups into the agarose molecules (Shillito *et al.,* 1983). Another advantage of agarose over agar lies in the removal of toxic components of agar during its preparation. Bolandi *et al.* (1999) preferred to embed protoplasts in agarose, rather than use liquid medium, because in agarose the semi-solid matrix applies a direct pressure on the plasma membrane of the protoplasts. They mixed protoplasts of sunflower with 0.5% agarose, pipetted 50 μl droplets of the mixture into Petri dishes and covered them with a thin layer of culture medium. A similar method was used to culture protoplasts of *Dioscorea* by Tor *et al.* (1999). The use of droplets has the advantage that a high plating density can be achieved in the droplets, while exposing the protoplasts to a larger reservoir of culture medium in the liquid phase. Bishoi *et al.* (2000) initially cultured anthers of Basmati rice on liquid medium, then used 1.0% agarose to culture calli derived from microspores.

6.2.2. Gellan gum

Gellan gum is a widely used gelling agent in plant tissue culture, that is marketed under various trade names including Gelrite, Phytagel and Kelcogel. It is an exopolysaccharide that encapsulates cells of the bacterium *Sphingomonas paucimobilis* (= *Auromonas elodea* = *Pseudomonas elodea*), from which it is obtained by industrial fermentation. The structure, physico-chemical properties and the rheology of solutions of gellan gum and related polysaccharides has been reviewed by Banik *et al.* (2000). Gellan gum consists of a linear repeating tetrasaccharide of D-glucose, D-glucuronic acid, D-glucose and L-rhamnose. Heating solutions of gellan gum in solutions that contain cations, such as K^+, Ca^{2+}, Mg^{2+}, causes the polysaccharide to form a gel in which the polymers form a half-staggered parallel double helix. The commercial deacetylated and purified polysaccharide forms a firm non-elastic gel. The gel sets rapidly at a temperature, determined by the concentrations of the polysaccharide and the cation, which varies between 35-50 °C (Kang *et al.,* 1982). The commercial product contains significant quantities of potassium, sodium, calcium and magnesium (Pasqualetto *et al.,* 1988a,b; Scherer *et al.,* 1988) but is said to be free of the organic impurities found in agar. It is unclear whether or not these cations remain fully available to plant cultures. Some researchers (Gawel *et al.,* 1986; 1990; Trolinder and Goodin, 1987; Umbeck *et al.,* 1987) add an extra 750 mg l^{-1} $MgCl_2$ to a medium containing MS salts to aid the gelling of 1.6% Gelrite. In most other reports on the use of Gelrite, cations in the medium have been sufficient to produce a gel.

Beruto *et al.* (1995) found that 0.12 % Gelrite and 0.7% Bacto agar have equivalent matric potential and support equivalent adventitious regeneration in leaf discs of sugarbeet. As gellan gum is used in lower concentration than agar, the cost per litre of medium is less. It produces a clear gel in which plant tissues can be more easily seen and microbial

contamination more easily detected than in agar gels. It has proved to be a suitable gelling agent for tissue cultures of many herbaceous plants and there are reports of its successful use for callus culture, the direct and indirect formation of adventitious organs and somatic embryos, shoot culture of herbaceous and semi-woody species and the rooting of plantlets. In most cases the results have been as good as, and sometimes superior to, those obtainable on agar-solidified media. Anders *et al.* (1988) described the regeneration of greater numbers of plants from of sugar cane on Gelrite than on the most productive brand of agar, and Koetje *et al.* (1989) obtained more somatic embryos from callus cultures of rice on media solidified with Gelrite than with Bacto-agar. Shoot cultures, particularly of some woody species, are liable to become hyperhydric if Gelrite, like agar, is used at too low a concentration. There are sharp differences in the response of different species to concentration of Gelrite. Turner and Singha (1990) found the highest rate of shoot proliferation in *Geum* occurred on 0.2% and in *Malus* on 0.4%. Garin *et al.* (2000) obtained more mature somatic embryos of *Pinus strobus* on gellan gum at 1% concentration of than at 0.6%. Pasqualetto *et al.* (1986a,b) used mixtures of Gelrite (0.1-0.15%) and Sigma @ agar (0.2-0.3%) to prevent the hyperhydricity that occurred in shoot cultures of *Malus domestica* 'Gala' on media solidified with Gelrite alone. Nairn (1988) used a mixture of Gelrite (0.194%) and Difco 'Bacto' agar (0.024%) to prevent the hyperhydricity that occurred in shoot cultures of *Pinus radiata* on medium gelled with 0.2% Gelrite alone.

6.2.3. Alginates

Alginic acid is a binary linear heteropolymer 1,4-β-D-mannuronic acid and 1,4-α-L-guluronic acid (Larkin *et al.,* 1988). It is extracted from various species of brown algae. When the sodium salt is exposed to calcium ions, gelation occurs. Alginates are widely used for protoplast culture and to encapsulate artificial seed.

Protoplasts embedded in beads or thin films of alginate can plated densely while yet exposed to a large pool of medium that dilutes inhibitors and toxic substances. Embedded protoplasts can be surrounded by nurse cells, either free in the surrounding medium or separated by filters or membranes. Alginate has the advantages over agarose that protoplasts do not have to be exposed to elevated temperatures when they are mixed with the gelling agent and the gel can be liquified by adding sodium citrate, releasing protoplasts or cell colonies for transfer to other media. The method of embedding protoplasts in beads as employed by Larkin *et al.* (1988) involved mixing the protoplast suspensions with an aqueous solution of sodium alginate and dropping it, through a needle, into a solution of calcium chloride. Beads containing the protoplasts were formed when the alginate made contact with the calcium ions. Beads with a final concentration of 1.5% sodium alginate were washed and cultured in liquid medium on an orbital shaker. Protoplasts may also be captured in thin layers of alginate (Dovzhenko *et al.,* 1998).

Synthetic seeds (synseeds, somatic seeds) encapsulated in alginate (Fig. 4.4), can be prepared from somatic embryos (Timbert *et al.,* 1995), shoot tips (Maruyama *et al.,* 1998), apical and axillary buds (Piccioni and Standardi, 1995), single nodes (Piccioni, 1997), and cell aggregates from hairy roots (Repunte *et al.,*1995). The uses of sythetic seeds include direct planting into soil, storage of tissues and transfer of materials between laboratories under sterile conditions. The methods of encapsulation of somatic embryos of carrot were described by Timbert *et al.* (1995). Torpedo-shaped embryos were mixed with a 1% sodium alginate solution. The mixture was dropped into a solution 100 mM calcium chloride in 10% sucrose. The beads (3-3.5 mm in diameter) were then rinsed in a 10% sucrose solution.

6.2.4. Starch

Sorvari (1986a,c) found that plantlets formed in higher frequencies in anther cultures of barley on a medium solidified with 5% corn starch or barley starch rather than with agar. Corn starch only formed a weak gel and it was necessary to place a polyester net on its surface to prevent the explants from sinking. Sorvari (1986b) found that it took 5-14 weeks for adventitious shoots to form on potato discs on agar-solidified medium but only 3 weeks on medium containing barley starch. Henderson and Kinnersley (1988) found that embryogenic carrot callus cultures grew slightly better on media gelled with 12% corn starch than on 0.9% Difco `Bacto' agar.

6.2.5. 'Kappa'--carrageenan

Carrageenan is a product of sea weeds of the genus *Euchema* and the kappa form has strong gelling properties. Like gellan gum, kappa-carrageenan requires the presence of cations for gelation. In Linsmaier and Skoog (1965) medium at 0.6% w/v, the gel strength was slightly less than that of 0.2% Gelrite or 0.8% of extra pure agar (Ichi *et al.,*

1986). Chauvin *et al.* (1999) found that regeneration from cultures of tulip, gladiolus and tobacco shoots was possible in the presence of 200 mg l^{-1} kanamycin, whereas in several other gelling agents a concentration of 100 mg l^{-1} inhibited regeneration.

6.2.6. Pectins

A mixture of pectin and agar can be a less expensive substitute for agar. For example, a semi-solid medium consisting of 0.2% agar plus 0.8-1.0% pectin, was employed for shoot culture of strawberry and some other plants (Zimmerman, 1979).

6.2.7. Other gelling agents

Battachary *et al.* (1994) found sago (from *Metroxylon sagu*) and isubgol (from *Plantago ovata*) were satisfactory substitutes for agar at, respectively, one eighth and one tenth of the cost of Sigma purified agar A7921.

6.3. POROUS SUPPORTS

Aeration of the tissues on a porous substrate is usually better than it would be in agar or static liquid.

Chin *et al.* (1988) used a buoyant polypropylene membrane floated on top of a liquid medium to culture cells and protoplasts of *Asparagus*. The membrane (Celgard 3500, Questar Corp., Charlotte, N.C.) has a pore size of 0.04 mm and is autoclavable. Conner and Meredith (1984) found that cells grew more rapidly on filter papers laid over polyurethane foam pads saturated with medium than on agar. Young *et al.* (1991) supported shoots of tomato over liquid culture medium on a floating microporous polypropylene membrane and entrained the growing shoots through polypropylene netting. They reported opportunities for the development of this method for mechanisation by mass handling. Membrane rafts were also used by Teng (1997) and Watad *et al.* (1995,1996).

Cheng and Voqui (1977) and Cheng (1978) used polyester fleece to support cultures of Douglas fir that were irrigated with liquid media in Petri dishes. Plantlets that were regenerated from cotyledon explants were cultured on 3 mm-thick fabric. When a protoplast suspension was dispersed over 0.5 mm-thick fabric, numerous colonies were produced in 12 days, whereas in the absence of the support, cell colonies failed to proliferate beyond the 20 cell stage. A major advantage in using this type of fabric support is that media can be changed simply and quickly without disturbing the tissues. The system has also been used for protoplast culture of other plants (e.g. by Russell and McCown, 1986).

Heller and Gautheret (1949) found that tissues could be satisfactorily cultured on pieces of ashless filter paper moistened by contact with liquid medium. Very small explants, such as meristem tips, that might be lost if placed in a rotated or agitated liquid medium, can be successfully cultured if placed on an M-shaped strip of filter paper (sometimes called a ‘Heller’ support). When the folded paper is placed in a tube of liquid medium, the side arms act as wicks (Goodwin, 1966). This method of support ensures excellent tissue aeration but the extra time required for preparation and insertion has meant that paper wicks are only used for special purposes such as the initial cultural stages of single small explants which are otherwise difficult to establish. Whether explants grow best on agar or on filter paper supports, varies from one species of plant to another. Davis *et al.* (1977) found that carnation shoot tips grew less well on filter paper bridges than on 0.6% agar but axillary bud explants of *Leucospermum* survived on bridges but not on agar.

Paper was also used in the construction of plugs (marketed by Ilacon Ltd, Tonbridge, UK TN9 1NR) known as Sorbarods (Roberts and Smith, 1990). These are cylindrical (20 mm in length and 18 mm in diameter) and consist of cold-crimped cellulose, longitudinally folded, wrapped in cellulose paper. The plug has a porosity (total volume – volume of cellulose) of 94.2% and high capillarity, so that the culture medium is efficiently drawn up into the plug, leaving the sides of the plug in direct contact with air. Roots permeate the plugs and are protected by the cellulose during transfer to soil. Plantlets of chrysanthemum in Sorbarods formed longer stems, larger leaves, more roots, and developed greater fresh mass, dry weights and fresh to dry mass ratios than plantlets in agar-solidified medium (Roberts and Smith, 1990). The greater fresh to dry mass ratio indicates that contact with liquid medium led to greater hydration of tissues. This was subsequently controlled by the inclusion of a growth retardant, paclobutrazol (1 mg l^{-1}), in the culture medium (Smith *et al.,* 1990a). Other porous materials that have been used to support plant growth include rockwool (Woodward *et al.,* 1991; Tanaka *et al.,* 1991), polyurethane foam (Gutman and Shiryaeva, 1980; Scherer *et al.,* 1988), vermiculite (Kirdmanee *et al.,* 1995), a mixture of vermiculite and Gelrite (Jay-Allemand *et al.,* 1992).

Afreen-Zobayed *et al.* (2000) cultured sweet potato, on sugar-free medium in autotrophic conditions, on mixtures of paper pulp and vermiculite in various proportions. Optimal growth was obtained on a mixture containing 70% paper pulp. On this

mixture, the fresh mass of plantlets was greater by a factor of 2.7 than on agar-solidified medium. Mixtures of paper pulp and vermiculite, in unspecified proportions, are prepared in a commercial product known as Florialite (Nisshinbo Industries, Inc., Tokyo). Afreen-Zobayed *et al.* (1999) found that growth rates of plantlets of sweet potato grown autotrophically on Florialite were greater, in ascending order, on agar, gellan gum, vermiculite, Sorbarods and Florialite (best). The dry mass of leaves and roots were greater by factors of 2.9 and 2.8, respectively, on Florialite than on an agar matrix. These authors observed that roots spread better in Florialite than in Sorbarods. They attributed this to the net-like orientation of fibers in Florialite that contrasted with the vertical orientation of fibres in Sorbarods. Ichimura and Oda (1995) found three substances that stimulated plant growth in extracts of paper pulp. Each was characterised by low molecular weight and high polarity. It is possible that these substances contribute to the superior growth observed on substrates containing paper pulp.

6.3.1. Opportunities for improved ventilation and photoautotrophy

When plantlets are cultured in vessels containing air at a relative humidity (RH) of less than 100%, transpiration occurs which is an important factor in reducing hyperhydricity (Gribble, 1999). Relative humidity in culture vessels can be reduced through ventilation, but gelled substrates are then unsuitable because the absorbance of water by the roots of a transpiring plant is impeded by the gel's low hydraulic conductivity (Fujiwara and Kozai, 1995) and this increases as the gel dries. Thus a common feature of studies using ventilated vessels has been the use of liquid medium supported by porous materials.

For example, when plantlets of chrysanthemum were grown in Sorbarods in a culture vessel with air 94% RH, a reduction in the tissue hydration was indicated by a significantly lower fresh to dry mass ratio than at 100% RH (Smith *et al.,* 1990b) and increases in stem length and leaf area. In this investigation, the RH was reduced to 94% by gaseous diffusion through a gas-permeable membrane that covered holes drilled in the lid of the culture vessel. The use of such ventilated culture vessels can significantly improve plant growth by reducing hyperhydration and facilitating the movement of solutes to the leaves in the transpiration stream. It also provides opportunities for photoautotrophic growth in sugar-free media. When plantlets are cultured in closed vessels, carbon dioxide concentrations fall to low levels in the light period, as Kozai and Sekimoto (1988) demonstrated in cultures of strawberry plants. Photosynthesis requires an adequate supply of carbon dioxide and suitable lighting. Adequate concentrations of carbon dioxide for photoautotrophy can be maintained in ventilated culture vessels with (Afreen-Zobayed *et al.,* 1999, 2000) or without (Horan *et al.,* 1995) elevated levels of carbon dioxide in the atmosphere outside the culture vessel. Adequate lighting can be achieved under lights delivering a photosynthetic photon flux of 150 $\mu mol\ m^{-2}\ s^{-1}$ in a culture room (Afreen-Zobayed *et al.,* 1999, 2000) or in day-light in a greenhouse (Horan *et al.,* 1995). The environmental requirements of photoautotrophy *in vitro* have been reviewed by Jeong *et al.* (1995) and its advantages have been described by Kozai *et al.* (1995).

6.4. IMMOBILISED CELLS

Yields of secondary metabolites are usually greater in differentiated, slow-growing cells than in fast growing, undifferentiated cells. By immobilising cells in a suitable matrix, their rate of growth can be slowed and the production of secondary products enhanced. Several ingenious methods of immobilisation have been employed. Examples include immobilization in spirally wound cotton (Choi *et al.* (1995), glass fibre fabric reinforced with a gelling solution of hybrid SiO_2 precursors (Campostrini *et al.,* 1996), loofa sponge and polyurethane foam (Liu *et al.,* 1999) and alginate beads (Serp *et al.,* 2000).

REFERENCES

ADRIAN J. & ASSOUMANI M. 1983 Gums and hydrocolloids in nutrition. pp. 301-333 in Rechcigl M. (ed.) 1983 *CRC Handbook of Nutritional Supplements* Vol. II. *Agricultural Use*. CRC Press Inc. Baton Rouge.

AFREEN-ZOBAYED F., ZOBAYED S.M.A., KUBOTA C., KOZAI T. & HASEGAWA O. 2000 A combination of vermiculite and paper pulp supporting material for the photoautotrophic micropropagation of sweet potato. Plant Sci. 157, 225-231.

AFREEN-ZOBAYED F., ZOBAYED S.M.A., KUBOTA C., KOZAI T. & HASEGAWA O. 1999 Supporting material affects the growth and development of *in vitro* sweet potato plantlets cultured photoautotrophically. In Vitro Cell. Dev. – Pl. 35, 470-474.

AHLOOWALIA B.S. & MARETZKI A. 1983 Plant regeneration via somatic embryogenesis in sugarcane. Plant Cell Rep. 2, 21-25.

ALBRECHT C. 1986 Optimization of tissue culture media. Comb. Proc. Int. Plant Prop. Soc. 1985, 35, 196-199.

ALONI R. 1980 Role of auxin and sucrose in the differentiation of seive and tracheary elements in plant tissue cultures. Planta 150, 255-263.

AMADOR A.M. & STEWART K.A. 1987 Osmotic potential and pH of fluid drilling gels as influenced by moisture loss and incorporation of growth regulators. J. Am. Soc. Hortic. Sci. 112, 26-28.

ANDERS J., LARRABEE P.L. & FAHEY J.W. 1988 Evaluation of gelrite and numerous agar sources for *in vitro* regeneration of sugarcane. HortScience 23, 755.

ANDERSON W.C. 1975 Propagation of rhododendrons by tissue culture. I. Development of a culture medium for multiplication of shoots. Comb. Proc. Int. Plant Prop. Soc. 25, 129-135.

ANDERSON W.C. 1978 Tissue culture propagation of rhododendrons. In Vitro 14, 334.

ANDERSON W.C. 1980 Tissue culture of red raspberries. pp. 27-34 in *Proceedings of the Conference on Nursery Production of Fruit Plants –Applications and Feasibility*. U.S.D.A., A.R.S., ARR-NE-11.

ANON (RESEARCH GROUP 301) 1976 A sharp increase of the frequency of pollen plant induction in wheat with potato medium. (Chinese) Acta Genet. Sin. 3, 25-31.

ANON 1980 Ann. Rep. and Accounts, British Petroleum Co. Ltd. 1979.

ANTHONY P., DAVEY M.R., POWER J.B. & LOWE K.C., 1995 An improved protocol for the culture of cassava leaf protoplasts. Plant Cell Tissue Organ Cult. 42, 299-302.

ANTHONY P., DAVEY M.R., POWER J.B. & LOWE K.C. 1997 Enhanced mitotic division of cultured *Passiflora* and *Petunia* protoplasts by oxygenated perfluorocarbon and haemoglobin. Biotechnol. Tech. 11, 581-584.

ARDITTI J. & ERNST R. 1984 Physiology of germinating orchid seeds. pp. 177-222 in Arditti J (ed.) 1984 *Orchid Biology - Reviews and Perspectives III*. Comstock Publishing, Cornell Univ. Press, Ithaca, London.

ARDITTI J. 1979 Aspects of the physiology of orchids. Adv. Bot. Res. 7, 421-655.

ARNOW P., OLESON J.J. & WILLIAMS J.H. 1953 The effect of arginine in the nutrition of *Chlorella vulgaris*. Am. J. Bot. 40, 100-104.

ASANO Y., KATSUMOTO H., INOKUMA C., KANEKO S., ITO Y. & FUJIIE A. 1996 Cytokinin and thiamine requirements and stimulative effects of riboflavin and α-ketoglutaric acid on embryogenic callus induction from the seeds of *Zoysia japonica* Steud. J. Plant Physiol. 149, 413-417.

ASHER C.J. 1978 Natural and synthetic culture media for spermatophytes. pp. 575-609 in Recheige M. Jr. (ed.). CRC *Handbook Series in Nutrition and Food*. Section G., *Culture Media and Food Supplements. Diets Vol 3.*

ATTREE S.M., MOORE D., SAWHNEY V.R. & FOWKE L.C. 1991 Enhanced maturation and desiccation tolerance of white spruce [*Picea glauca* (Moench.) Voss] somatic embryos: effects of a non-plasmolysing water stress and abscisic acid. Ann. Bot. 68, 519-525.

AYABE S., UDAGAWA A. & FURUYA T. 1988 Stimulation of chalcone synthase activity by yeast extract in cultured *Glycorrhiza echinata* cells and 5-deoxyflavone formation by isolated protoplasts. Plant Cell Rep. 7, 35-38.

BAGGA S., DAS R. & SOPORY S.K. 1987 Inhibition of cell proliferation and glyoxalase-1 activity by calmodulin inhibitors and lithium in *Brassica oleracea*. J. Plant Physiol. 129, 149-153.

BALL E. 1953 Hydrolysis of sucrose by autoclaving media, a neglected aspect in the culture of plant tissues. B. Torrey Bot. Club 80, 409-411.

BANIK R.M., KANARI B. & UPADHYAY S.N., 2000. Exopolysaccharide of the gellan family: prospects and potential. World J. Microb. Biot. 16, 407-414.

BARGHCHI M. 1988 Micropropagation of *Alnus cordata* (Loisel.) Loisel. Plant Cell Tissue Organ Cult. 15, 233-244.

BARG R. & UMIEL N. 1977 Effects of sugar concentration on growth, greenery and shoot formation in callus cultures of four genetic lines of tobacco. Z. Pflanzenphysiol. 81, 161-166.

BARLASS M., GRANT W.J.R. & SKENE K.G.M. 1980 Shoot regeneration *in vitro* from native Australian fruit-bearing trees – Quandong and Plum bush. Aust. J. Bot. 28, 405-409.

BARWALE U.B., KERNS H.R. & WIDHOLM J.M. 1986 Plant regeneration from callus cultures of several soybean genotypes via embryogenesis and organogenesis. Planta 167, 473-481.

BEHREND J. & MATELES R.I. 1975 Nitrogen metabolism in plant cell suspension cultures. I. Effect of amino acids on growth. Plant Physiol. 56, 584-589.

BERGHOEF J. & BRUINSMA J. 1979 Flower development of *Begonia franconis* Liebm. IV. Adventitious flower bud formation in excised inflorescence pedicels *in vitro*. Z. Pflanzenphysiol. 94, 407- 416.

BERGMANN L. 1967 Wachstum gruner Suspensionskulturen von *Nicotiana tabacum* Var. 'Samsun' mit CO_2 als Kohlenstoffquelle. Planta 74, 243-249.

BERUTO D., BERUTO M., CICCARELLI C. & DEBERGH P. 1995 Matric potential evaluations and measurements for gelled substrates. Physiol. Plant. 94, 151-157.

BHATTACHARYA P., DEY S. & BHATTACHARYA B.C. 1994 Use of low-cost gelling agents and support matrices for industrial-scale plant tissue culture. Plant Cell Tissue Organ Cult. 37, 15-23.

BHOJWANI S.S. & RAZDAN M.K. 1983 *Plant Tissue Culture: Theory and Practice.* Elsevier Science Publishers B.V., Amsterdam.

BIONDI S. & THORPE T.A. 1982 Growth regulator effects, metabolite changes and respiration during shoot initiation in cultured cotyledon explants of *Pinus radiata*. Bot. Gaz. 143, 20-25.

BISHNOI U.S., JAIN R.K., GUPTA K.R., CHOWDHURY V.K. & CHOWDHURY J.B. 2000 High frequency androgenesis in *indica* x Basmati rice hybrids using liquid culture media. Plant Cell Tissue Organ Cult. 61, 153-159.

BISWAS G.C.G. & ZAPATA F.J. 1993 High-frequency plant regeneration from protoplasts of *indica* rice (*Oryza sativa* L.) using maltose. J. Plant Physiol. 141, 470-475.

BOLANDI A.R., BRANCHARD M., ALIBERT G., SERIEYS H. & SARRAFI A. 1999 Cytoplasmic-nuclear interaction and medium effect for protoplast culture in sunflower. Plant Cell Tissue Organ Cult. 57, 189-193.

BOLL W.G. & STREET H.E. 1951 Studies on the growth of excised roots. 1. The stimulatory effect of molybdenum and copper on the growth of excised tomato roots. New Phytol. 50, 52-75.

BONNER J. & ADDICOTT F. 1937 Cultivation *in vitro* of excised pea roots. Bot. Gaz. 99, 144-170.

BONNER J. & DEVIRIAN P.S. 1939 Growth factor requirements of four species of isolated roots. Am. J. Bot. 26, 661-665.

BONNER J. & DEVIRIAN P.S. 1939 Growth factor requirements of four species of isolated roots. Am. J. Bot. 26, 661-665.

BONNER J. 1937 Vitamin B1, a growth factor for higher plants. Science 85, 183-184.

BONNER J. 1938a Thiamin (Vitamin B1) and the growth of roots: the relation of chemical structure to physiological activity. Am. J. Bot. 25, 543-549.

BONNER J. 1940a Specificity of nicotinic acid as a growth factor for isolated pea roots. Plant Physiol. 15, 553-557.

BORGES M., MENESES S., VAZQUEZ J., GARCIA M., AGUILERA N., INFANTE Z., RODRIGUEZ A. & FONSECA M. 2003 *In vitro* conservation of *Dioscorea alata* L. germplasm by slow growth. Plant Gen. Res. Newsl. 133, 8-12.

BORKIRD C. & SINK K.C. 1983 Medium components for shoot cultures of chlorophyll-deficient mutants of *Petunia inflata*. Plant Cell Rep. 2, 1-4.

BÖTTGER M. & LUTHEN H. 1986 Possible linkage between NADH-oxidation and proton secretion in *Zea mays* L. roots. J. Exp. Bot. 37, 666-675.

BOZHKOV P.V. & VON ARNOLD S. 1998 Polyethylene glycol promotes maturation but inhibits further development of *Picea abies* somatic embryos. Physiol. Plant. 104, 211-224.

BRETZLOFF C.W. Jr. 1954 The growth and fruiting of *Sordaria fimicola*. Am. J. Bot. 41, 58-67.

BRIDSON E.Y. 1978 Diets, culture media and food supplements. pp. 91-281 in Rechcigl M. Jr. (ed.) *CRC Handbook Series in Nutrition and Food*. Section G. Vol.3.

BRINK R.A., COOPER D.C. & AUSHERMAN L.E. 1944 A hybrid between *Hordeum jubatum* and *Secale cereale*. J. Hered. 35, 67-75.

BROWN D.C.W. & THORPE T.A. 1982 Mitochondrial activity during shoot formation and growth in tobacco callus. Physiol. Plant. 54, 125-130.

BROWN D.C.W. & THORPE T.A. 1980 Changes in water potential and its components during shoot formation in tobacco callus. Physiol. Plant. 49, 83-87.

BROWN D.C.W., LEUNG D.W.M. & THORPE T.A. 1979 Osmotic requirement for shoot formation in tobacco callus. Physiol. Plant. 46, 36-41.

BROWN C., BROOKS E.J., PEARSON D. & MATHIAS R.J. 1989 Control of embryogenesis and organogenesis in immature wheat embryo callus using increased medium osmolarity and abscisic acid. J. Plant Physiol. 133, 727-733.

BURNET G. & IBRAHIM R.K. 1973 Tissue culture of *Citrus* peel and its potential for flavonoid synthesis. Z. Pflanzenphysiol. 69, 152-162.

BURSTROM H. 1957 Root surface development, sucrose inversion and free space. Physiol. Plant. 10, 741-751.

BUTCHER D.N. & STREET H.E. 1964 Excised root culture. Bot. Rev. 30, 513-586.

BUTENKO R.G., LIPSKY A.K.H., CHERNYAK N.D. & ARYA H.C. 1984 Changes in culture medium pH by cell suspension cultures of *Dioscorea deltoidea*. Plant Sci. Lett. 35, 207-212.

CALLEBAUT A. & MOTTE J.-C. 1988 Growth of cucumber cells in media with lactose or milk whey as carbon source. Plant Cell Rep. 7, 162- 165.

CAMPOSTRINI R., CARTURAN G., CANIATO R., PIOVAN A., FILIPPINI R., INNOCENTI G. & CAPPELLETTI E.M. 1996 Immobilization of plant cells in hybrid sol-gel materials. J. Sol-Gel Sci. Techn. 7, 87-97.

CANHOTO J.M. & CRUZ G.S. 1994 Improvement of somatic embryogenesis in *Feijoa sellowiana* Berg (Myrtaceae) by manipulation of culture media composition. In Vitro Cell. Dev. – Pl. 30, 21-25.

CAPLIN S.M. & STEWARD F.C. 1948 Effects of coconut milk on the growth of explants from carrot root. Science 108, 655-657.

CARCELLER M., DAVEY M.R., FOWLER M.W. & STREET H.E. 1971 The influence of sucrose, 2,4-D and kinetin on the growth, fine structure and lignin content of cultured sycamore cells. Protoplasma 73, 367-385.

CARIMI F., DE PASQUALE F. & CRESCIMANNO F.G. 1999 Somatic embryogenesis and plant regeneration from pistil thin cell layers of Citrus. Plant Cell Rep. 18, 935-940.

CARIMI F., DE PASQUALE F. & PUGLIA A.M. 1998 *In vitro* rescue of zygotic embryos of sour orange, *Citrus aurantium* L., and their detection based on RFLP analysis. Plant Breeding 117, 261-266.

CARPITA N., SABULARSE D., MONTEZINOS D. & DELMER D.P. (1979) Determination of the pore size of cell walls of living plant cells. Science 205,1144-1147.

CHANDLER M.T., TANDEAU DE MARSAC N. & KOUCHKOVSKY Y. 1972 Photosynthetic growth of tobacco cells in liquid suspensions. Can. J. Bot. 50, 2265-2270.

CHANDLER S.F., RAGOLSKY E., PUA E.-C. & THORPE T.A. 1987 Some morphogenic effects of sodium sulphate on tobacco callus. Plant Cell Tissue Organ Cult. 11, 141-150.

CHAUVIN J.E. & SALESSES G. 1988 Advances in chestnut micropropagation (*Castanea* sp.). Acta Hortic. 227, 340-345.

CHAUVIN J.E., MARHADOUR S., COHAT J. & LE NARD M. 1999 Effects of gelling agents on *in vitro* regeneration and kanamycin efficiency as a selective agent in plant transformation procedures. Plant Cell Tissue Organ Cult. 58, 213-217.

CHAZEN O., HARTUNG W. & NEUMANN P.M. 1995 The different effects of PEG 6000 and NaCl on leaf development are associated with differential inhibition of root water transport. Plant Cell Environ. 18, 727-735.

CHEE P.P. 1995 Stimulation of adventitious rooting of *Taxus* species by thiamine. Plant Cell Rep. 14, 753-757.

CHEE R.P., SCHULTHEIS J.R. & CANTCLIFFE D.J. 1990 Plant recovery from sweet potato somatic embryos. HortScience 25, 795-797.

CHENG T.-Y. & VOQUI T.H. 1977 Regeneration of Douglas fir plantlets through tissue culture. Science 198, 306-307.

CHENG T.-Y. 1978 Clonal propagation of woody species through tissue culture techniques. Comb. Proc. Int. Plant Prop. Soc. 28, 139-155.

CHEVRE A.-M., GILL S.S., MOURAS A. & SALESSES G. 1983 *In vitro* multiplication of chestnut. J. Hortic. Sci. 58, 23-29.

CHIEN Y.C. & KAO K.N. 1983 Effects of osmolality, cytokinin and organic acids on pollen callus formation in *Triticale* anthers. Can. J. Bot. 61, 639-641.

CHIN C. & MILLER D. 1982 Some characteristics of the phosphate uptake by *Petunia* cells. HortScience 17, 488.

CHIN C.-K., KONG Y. & PEDERSEN H. 1988 Culture of droplets containing asparagus cells and protoplasts on polypropylene membrane. Plant Cell Tissue Organ Cult. 15, 59-65.

CHOI H.J., TAO B.Y. & OKOS M.R. 1995 Enhancement of secondary metabolite production by immobilized *Gossypium arboreum* cells. Biotechnol. Progr. 11, 306-311.

CHONG C. & PUA E.-C. 1985 Carbon nutrition of Ottawa 3 apple rootstock during stages of *in vitro* propagation. J. Hortic. Sci. 60, 285-290.

CHONG C. & TAPER C.D. 1972 *Malus* tissue culture. I. Sorbitol (D-glucitol) as carbon source for callus initiation and growth. Can. J. Bot. 50, 1399-1404.

CHONG C. & TAPER C.D. 1974a *Malus* tissue cultures. II. Sorbitol metabolism and carbon nutrition. Can. J. Bot. 52, 2361-2364.

CHONG C. & TAPER C.D. 1974b Influence of light intensity on sorbitol metabolism, growth and chlorophyll content of *Malus* tissue cultures. Ann. Bot. 38, 359-362.

CHU C.-C., WANG C.-C., SUN C.-S., HSU C., YIN K.-C., CHU C.-Y. & BI F.-Y. 1975 Establishment of an efficient medium for anther culture of rice, through comparative experiments on the nitrogen sources. Sci.Sinica 18, 659-668.

CHUA S.E. 1966 Studies on tissue culture of *Hevea brasiliensis*. I. Role of osmotic concentration, carbohydrate and pH value in induction of callus growth in plumule tissue from *Hevea* seedling. J. Rubber Res. Inst. Malaya 19, 272-276.

CHUANG C.-C., OUYANG T.-W., CHIA H., CHOU S.-M. & CHING C.-K. 1978 A set of potato media for wheat anther

culture. pp. 51-56 in *Plant Tissue Culture.* Proceedings of the Peking Symposium 1978. Pitman, Boston, London, Melbourne.

CHUONG P.V. & BEVERSDORF W.D. 1985 High frequency embryogenesis through isolated microspore culture in *Brassica napus* L and *B. carinata* Braun. Plant Sci. 39, 219-226.

CLELAND R. 1977 The control of cell enlargement. pp. 101-115 in Jennings D.H. (ed.) S.E.B. Symp. 31. University Press, Cambridge.

COFFIN R., TAPER C.D. & CHONG C. 1976 Sorbitol and sucrose as carbon source for callus culture of some species of the *Rosaceae*. Can. J. Bot. 54, 547-551.

CONNER A.J. & FALLOON P.G. 1993 Osmotic versus nutritional effects when rooting *in vitro* asparagus minicrowns on high sucrose. Plant Sci. 89, 101-106.

CONNER A.J. & MEREDITH C.P. 1984 An improved polyurethane support system for monitoring growth in plant cell cultures. Plant Cell Tissue Organ Cult. 3, 59-68.

CONRAD P.L., CONRAD J.M., DURBIN R.D. & HELGESON J.P. 1981 Effects of some organic buffers on division of protoplast-derived cells and plant regeneration. Plant Physiol. 67, Suppl., 116.

COOPER A. 1979 *The ABC of NFT*. Grower Books, London. ISBN 0 901 361224.

COPPING L.G. & STREET H.E. 1972 Properties of the invertases of cultured sycamore cells and changes in their activity during culture growth. Physiol. Plant. 26, 346-354.

CULAFIC L., BUDIMIR S., VUJICIC R. & NESKOVIC M. 1987 Induction of somatic embryogenesis and embryo development in *Rumex acetosella* L. Plant Cell Tissue Organ Cult. 11, 133-139.

DABIN P. & BEGUIN F. 1987 Somatic embryogenesis in *Fuchsia*. Acta Hortic. 212, 725-726.

DAIGNY G., PAUL H. & SANGWAN-NORRELL B.S. 1996 Factors influencing secondaru somatic embryogenesis in *Malus* x *domestica* Borkh. (cv 'Gloster 69'). Plant Cell Rep. 16, 153-157.

DALTON C.C., IQBAL K. & TURNER D.A. 1983 Iron phosphate precipitation in Murashige and Skoog media. Physiol. Plant. 57, 472-476.

DAMIANO C., CURIR P. & COSMI T. 1987 Short note on the effects of sugar on the growth of *Eucalyptus gunnii in vitro*. Acta Hortic. 212, 553-556.

DANTU P.K. & BHOJWANI S.S. 1995 *In vitro* corm formation and field evaluation of corm-derived plants of *Gladiolus*. Sci. Hortic. 61, 115-129.

DAS R., BAGGA S. & SOPORY S.K. 1987 Involvement of phosphoinositides, calmodulin and glyoxidase-1 in cell proliferation in callus cultures of *Amaranthus paniculatus*. Plant Sci. 53, 45-51.

DAS T., MITRA G.C. & CHATTERJEE A. 1995 Microprop-agation of *Citrus sinensis* var. mosambi: an important scion. Phytomorph. 45, 57-64.

DAVIS M.J., BAKER R. & HANAN J.J. 1977 Clonal multi-plication of carnation by micropropagation. J. Am. Soc. Hortic. Sci. 102, 48-53.

DAY D. 1942 Thiamin content of agar. B. Torrey Bot. Club 69, 11-20.

DE BRUYN M.H. AND FERREIRA D.I. 1992 *In vitro* corm production of *Gladiolus dalenii* and *G. tristis*. Plant Cell Tissue Organ Cult. 31, 123-128.

DE CAPITE L. 1948 pH of nutritive solutions and its influence on the development of plants. Ann. Fac. Agrar. Univ. Perugia 5, 135-145.

DE CAPITE L. 1952a Combined action of p-aminobenzoic acid and indoleacetic acid on the vascular parenchyma of Jerusalem artichoke cultured *in vitro*. Compt. Rend. Soc. Biol. 146, 863-865.

DE CAPITE L. 1952b Action of p-aminophenylsulphonamide and p-amino-benzoic acid on the vascular parenchyma of Jerusalem artichoke and of crown-gall tissue of salsify. Compt. Rend. Acad. Sci. Paris 234, 2478-2480.

DE FOSSARD R.A., MYINT A. & LEE E.C.M. 1974 A broad spectrum tissue culture experiment with tobacco (*Nicotiana tabacum*) pith tissue callus. Physiol. Plant. 31, 125-130.

DE JONG A.W. & BRUINSMA J. 1974 Pistil development in *Cleome* flowers III. Effects of growth-regulating substances on flower buds of *Cleome iberidella* Welv. ex Oliv. grown *in vitro*. Z. Pflanzenphysiol. 73, 142-151.

DE JONG A.W., SMIT A.L. & BRUINSMA J. 1974 Pistil development in *Cleome* flowers II. Effects of nutrients on flower buds of *Cleome iberidella* Welv. ex Oliv. grown *in vitro*. Z. Pflanzenphysiol. 72, 227-236.

DE KLERK G.J., HANECAKOVA J. & JASIK J. 2007 Effect of medium-pH on adventitious root formation from thin stem disks cut from apple microshoots. Plant Cell Tissue Organ Cult. (in press)

DE KLERK-KIEBERT Y.M. & VAN DER PLAS L.H.W. 1985 Relationship of respiratory pathways in soybean cell suspensions to growth of the cells at various glucose concentrations. Plant Cell Tissue Organ Cult. 4, 225-233.

DE KRUIJFF E. 1906 Composition of coconut water and presence of diastase in coconuts. B. Dep. Agric. Indes Neerland. 4, 1-8.

DE PASQUALE F., CARIMI F. & CRESCIMANNO F.G. 1994 Somatic embryogenesis from styles of different cultivars of *Citrus limon* (L.) Burm. Aust. J. Bot, 42, 587-594.

DE PINTO M.C., FRANCIS D. & DE GARA D. 1999 The redox state of the ascorbate-dehydroascrobate pair as a specific sensor of cell division in tobacco BY-2 cells. Protoplasma 209, 90-97.

DEBERGH P.C. 1983 Effects of agar brand and concentration on the tissue culture medium. Physiol. Plant. 59, 270-276.

DEBERGH P., AITKEN-CHRISTIE J., COHEN D., GROUT B., VON ARNOLD S., ZIMMERMAN R. & ZIV M. 1992 Recon-sideration of the term 'vitrification' as used in micropropagation. Plant Cell Tissue Organ Cult. 30, 135-140.

DEBERGH P., HARBAOUI Y. & LEMEUR R. 1981. Mass propagation of globe artichoke (*Cyanara scolymus*): evaluation of different hypotheses to overcome vitrification with special reference to water potential. Physiol. Plant. 53, 181-187

DELBARRE A., MULLER P., IMHOFF V. & GUERN J. 1996 Comparison of mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells. Planta 198, 532-541.

DELFEL N.E. & SMITH L.J. 1980 The importance of culture con-ditions and medium component interactions on the growth of *Cephalotaxus harringtonia* tissue cultures. Planta Medica 40, 237-244.

DIGBY J. & SKOOG F. 1966 Cytokinin activation of thiamine biosynthesis in tobacco callus cultures. Plant Physiol. 41, 647-652.

DIJKEMA C., BOOIJ H., DE JAGER P.A., DE VRIES S.C., SCHAAFSMA T.J. & VAN KAMMEN A. 1990 Aerobic hexose metabolism in embryogenic cell lines of *Daucus carota* studied by ^{13}C NMR using ^{13}C-enriched substrates. pp. 343-348 in Nijkamp *et al.* (eds.) 1990 (q.v.).

DIX L. & VAN STADEN J. 1982 Auxin and gibberellin-like substances in coconut milk and malt extract. Plant Cell Tissue Organ Cult. 1, 239-245.

DOLEY D. & LEYTON L. 1970 Effects of growth regulating substances and water potential on the development of wound callus in *Fraxinus*. New Phytol. 69, 87-102.

DORAN P.M. 1993 Design of bioreactors for plant cells and organs. pp 116-169 in Fiechter A. (ed.) *Bioprocess design and control. Vol. 48* Springer-Verlag, Berlin. pp 116-169.

DOUGALL D.K. 1980 Nutrition and Metabolism. *Plant Tissue Culture as a Source of Biochemicals.* C.R.C. Press, Boca Raton, Florida.

DOUGALL D.K., WEYRAUCH K.W. & ALTON W. 1979 The effects of organic acids on growth and anthocyanin production by wild carrot cells growing on ammonia as a sole nitrogen source. In Vitro 15, 189-190 (Abst. 103).

DOUGLAS T.J., VILLALOBOS V.M., THOMPSON M.R. & THORPE T.A. 1982 Lipid and pigment changes during shoot-inititaion in cultured explants of *Pinus radiata*. Physiol. Plant. 55, 470-477.

DOVZHENKO A., BERGEN U. & KOOP H.U. 1998 Thin-alginate-layer technique for protoplast culture of tobacco leaf protoplasts: shoot formation in less than two weeks. Protoplasma 204, 114-118.

DREW R.A. & SMITH N.G. 1986 Growth of apical and lateral buds of pawpaw (*Carica papaya* L.) as affected by nutritional and hormonal factors. J. Hortic. Sci. 61, 535-543.

DREW R.A., MCCOMB J.A. & CONSIDINE J.A. 1993 Rhizogenesis and root growth of *Carica papaya* L. *in vitro* in relation to auxin sensitive phases and use of riboflavin. Plant Cell Tissue Organ Cult. 33, 1-7.

DRUART PH. 1988 Regulation of axillary branching in micropropagation of woody fruit species. Acta Hortic. 227, 369-380.

DUHAMET L. & MENTZER C. 1955 Essais d'isolement des substances excito-formatrices du lait de coco. Compt. Rend. Acad. Sci. Paris 241, 86-88.

DUNSTAN D.I. 1982 Transplantation and post-transplantation of micropropagated tree-fruit rootstocks. Comb. Proc. Int. Plant Prop. Soc., 1981 31, 39-44.

DUNSTAN W.R. 1906 Report on a sample of coconut "water" from Ceylon. Trop. Agric. (Ceylon) 26, 377-378.

DUNWELL J.M. 1981 Influence of genotype and environment on growth of barley *Hordeum vulgare* embryos *in vitro*. Ann. Bot. 48, 535-542.

EMONS A.M.C., SAMALLO-DROPPERS A. & VAN DER TOORN C. 1993 The influence of sucrose, mannitol, L-proline, abscisic acid and gibberellic acid on the maturation of somatic embryos of *Zea mays* L. from suspension cultures. J. Plant Physiol. 142, 597-604.

EARLE E.D. & TORREY J.G. 1965 Colony formation by isolated *Convolvulus* cells plated on defined media. Plant Physiol. 40, 520-528.

EDELMAN J. & HANSON A.D. 1972 Sucrose suppression of chlorophyll synthesis in carrot-tissue cultures. J. Exp. Bot. 23, 469-478.

EDWARDS K.J. & GOLDSMITH M.H.M. 1980 pH-dependent accumulation of indoleacetic acid by corn coleoptile sections. Planta 147, 457-466.

EINSET J.W. 1978 Citrus tissue culture - stimulation of fruit explant cultures with orange juice. Plant Physiol. 62, 885-888.

EL HINNAWIY E. 1974 Effect of some growth regulating substances and carbohydrates on chlorophyll production in *Melilotus alba* (Desr.) callus tissue cultures. Z. Pflanzenphysiol. 74, 95-105.

ERNER Y. & REUVENI O. 1981 Promotion of citrus tissue culture by citric acid. Plant Physiol. 67, Suppl., 27.

ERNER Y. & REUVENI O. 1981 Promotion of citrus tissue culture by citric acid. Plant Physiol. 67, Suppl., 27.

ERNST R. 1967 Effects of carbohydrate selection on the growth rate of freshly germinated *Phalaenopsis* and *Dendrobium* seed. Am. Orchid Soc. Bull. 36, 1068-1073.

ERNST R. 1974 The use of activated charcoal in asymbiotic seedling culture of *Paphiopedilum*. Am. Orchid Soc. Bull. 43, 35-38.

ERNST R., ARDITTI J. & HEALEY P.L. 1971 Carbohydrate physiology of orchid seedlings. II. Hydrolysis and effects of oligosaccharides. Am. J. Bot. 58, 827-835.

EVANS D.A., SHARP W.R. & PADDOCK E.F. 1976 Variation in callus proliferation and root morphogenesis in leaf tissue cultures of *Glycine max* strain T 219. Phytomorph. 26, 379-384.

FELLE H.H. 1998. The apoplastic pH of the *Zea mays* root cortex as measured with pH-sensitive microelectrodes: aspects of regulation. J. Exp. Bot. 49, 987-995.

FELLE H.H. 2001 pH: Signal and messenger in plant cells. Plant Biol. 3, 577-591.

FELLE H.H. & HANSTEIN S. 2002 The apoplastic pH of the substomatal cavity of *Vicia faba* leaves and its regulation responding to different stress factors. J. Exp. Bot. 53, 73-82.

FERGUSON J.D., STREET H.E. & DAVID S.B. 1958 The carbohydrate nutrition of tomato roots. V. The promotion and inhibition of excised root growth by various sugars and sugar alcohols. Ann. Bot. 22, 513-524.

FINDENEGG G.R., VAN BEUSICHEM M.L. & KELTJENS W.G. 1986 Proton balance of plants: physiological, agronomical and economical implications. Neth. J. Agric. Sci. 34, 371-379.

FINNIE S.J., POWELL W. & DYER A.F. 1989 The effect of carbohydrate composition and concentration on anther culture response in barley. Plant Breeding 103, 110-118.

FONNESBECH M. 1972 Organic nutrients in the media for the propagation of *Cymbidium in vitro*. Physiol. Plant. 27, 360-364.

FOX J.E. & MILLER C. 1959 Factors in corn steep water promoting growth of plant tissues. Plant Physiol. 34, 577-579.

FUGGI A., RUGANO V.M., VONA V. & RIGANO C. 1981 Nitrate and ammonium assimilation in algae cell-suspensions and related pH variations in the external medium, monitored by electrodes. Plant Sci. Lett. 23, 129-138.

FUJIWARA A. (ed.) 1982 *Plant Tissue Culture* 1982. Proc. 5th. Int. Cong. Plant Tiss. Cell Cult., Japan. Assoc. Plant Tissue Culture, Japan.

FUJIWARA K. & KOZAI T. 1995 Physical microenvironment and its effects. pp. 319-369 in Aitken-Christie J., Kozai T. and Smith M.L. (eds.) *Automation and Environmental Control in Plant Tissue Culture*. Kluwer Academic Publishers, The Netherlands.

FUKAMI T. & HILDEBRANDT A.C. 1967 Growth and chlorophyll formation in edible green plant callus tissues *in vitro* on media with limited sugar supplements. Bot. Mag. Tokyo 80, 199-212.

FURGUSON J.D. 1967 The nutrition of excised wheat roots. Physiol. Plant. 20, 276-284.

GALIBA G. & ERDEI L. 1986 Dependence of wheat callus growth, differentiation and mineral content on carbohydrate supply. Plant Sci. 45, 65-70.

GALIBA G. & YAMADA Y. 1988 A novel method for increasing the frequency of somatic embryogenesis in wheat tissue culture by NaCl and KCl supplementation. Plant Cell Rep. 7, 55-58.

GAMBORG O.L. & SHYLUK J.P. 1970 The culture of plant cells with ammonium salts as a sole nitrogen source. Plant Physiol. 45, 598-600.

GAMBORG O.L., CONSTABEL F. & SHYLUK J.P. 1974 Organogenesis in callus from shoot apices of *Pisum sativum*. Physiol. Plant. 30, 125-128.

GAMBORG O.L., MILLER R.A. & OJIMA K. 1968 Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151-158.

GAMBORG O.L., MILLER R.A. & OJIMA K. 1968 Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151-158.

GARIN E., BERNIER-CARDOU M. , ISABEL N., KLIMASZEWSKA K. & PLOURDE A. 2000 Effect of sugars, amino acids and culture technique on maturation of somatic

embryos of *Pinus strobus* on medium with two gellan gum concentrations. Plant Cell Tissue Organ Cult. 62, 27-37.

GASPAR T., KEVERS C., DEBERGH P., MAENE L., PAQUES M. & BOXUS P. 1987 Vitrification: morphological, physiological and ecological aspects. in Bonga J.M. and Durzan D.J. (eds.) *Cell and Tissue Culture in Forestry Vol. 1* Kluwer Academic Press Publ., Dordrecht.

GAUTHERET R.J. 1942 *Manuel Technique de Culture des Tissue Végétaux*. Masson et Cie, Paris.

GAUTHERET R.J. 1945 Une voie nouvelle en biologie végétale: la culture des tissus. Gallimard, Paris.

GAUTHERET R.J. 1948 Sur la culture indéfinie des tissus de *Salix caprea*. Compt. Rend. Soc. Biol. 142, 807.

GAWEL N.J., RAO A.P. & ROBACKER C.D. 1986 Somatic embryogenesis from leaf and petiole callus cultures of *Gossypium hirsutum* L. Plant Cell Rep. 5, 457-459.

GAWEL N.J. & ROBACKER C.D. 1990 Somatic embryogenesis in two *Gossypium hirsutum* genotypes on semi-solid versus liquid proliferation media. Plant Cell Tissue Organ Cult.23, 201-204.

GEORGE E.F., PUTTOCK D.J.M. & GEORGE H.J. 1987 *Plant Culture Media Vol.1*. Exegetics Ltd., Westbury, England.

GERRITS M. & DE KLERK G.J. 1992 Dry-matter partitioning between bulbs and leaves in plantlets of *Lilium speciosum* regenerated *in vitro*. Acta Bot. Neerl. 41, 461-468.

GLASSTONE S. 1946 *Textbook of Physical Chemistry.* Second Edition. MacMillan, London.

GOLDSCHMIDT E.E. 1976 Edogenous growth substances of citrus tissues. HortScience 11, 95-99.

GOOD N.E., WINGET G.D., WINTER W., CONNOLLY T.N., IZAWA S. & SINGH R.M. 1966 Hydrogen ion buffers for biological research. Biochemistry 5, 467-477.

GOODWIN P.B. 1966 An improved medium for the rapid growth of isolated potato buds. J. Exp. Bot. 17, 590-595.

GOPAL J., CHAMAIL A. & SARKAR D. 2002 Slow-growth *in vitro* conservation of potato germplasm at normal propagation temperature. Pot. Res. 45, 203-213.

GRANATEK C.H. & COCKERLINE A.W. 1978 Callus formation versus differentiation of cultured barley embryos: hormonal osmotic interactions. In Vitro 14, 212-217.

GRAY D.J., CONGER B.V. & SONGSTAD D.D. 1987 Desiccated quiescent somatic embryos of orchard grass for use as synthetic seeds. In Vitro Cell. Dev. 23, 29-32.

GRIBBLE K. 1999 The influence of relative humidity on vitrification, growth and morphology of *Gypsophila paniculata* L. Plant Growth Regul. 27, 179-188.

GROOTAARTS H., SCHEL J.H.N. & PIERIK R.L.M. 1981 The origin of bulblets formed on excised twin scales of *Nerine bowdenii* W. Watts. Plant Cell Tissue Organ Cult. 1, 39-46.

GROSS K.C., PHARR D.M. & LOCY R.D. 1981 Growth of callus initiated from cucumber hypocotyls on galactose and galactose-containing oligosaccharides. Plant Sci. Lett. 20, 333-341.

GUHA S. & JOHRI B.M. 1966 *In vitro* development of overy and ovule of *Allium cepa* L. Phytomorph. 16, 353-364.

GUHA S. & MAHESHWARI S.C. 1964 *In vitro* production of embryos from anthers of *Datura*. Nature 204, 497.

GUHA S. & MAHESHWARI S.C. 1967 Development of embryoids from pollen grains of *Datura in vitro*. Phytomorph. 17, 454-461.

GUNNING B.E.S. & STEER M.W. 1975 *Ultrastructure and the Biology of Plant Cells.* Arnold, London.

GUPTA P.K. & DURZAN D.J. 1985 Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). Plant Cell Rep. 4, 177-179.

GUPTA P.K., DANDEKAR A.M. & DURZAN D.J. 1988 Somatic proembryo formation and transient expression of a luciferase gene in Douglas fir and loblolly pine protoplasts. Plant Sci. 58, 85-92.

GUTMAN T.S. & SHIRYAEVA G.A. 1980 New method for growing cultures of isolated tissues of higher plants. Rastit. Resur. 16, 601-606.

HACKETT D.P. 1952 The osmotic change during auxin-induced water uptake by potato tissue. Plant Physiol. 27, 279-284.

HAKKAART F.A. & VERSLUIJS J.M.A. 1983 Some factors affecting glassiness in carnation meristem tip cultures. Neth. J. Plant Path. 89, 47-53.

HALPERIN W. & WETHERELL D.F. 1964 Adventive embryony in tissue cultures of the wild carrot, *Daucus carota*. Am. J. Bot. 51, 274-283.

HAMAOKA Y., FUJITA Y. & IWAI S. 1991 Effects of temperature on the mode of pollen development in anther culture of *Brassica campestris*. Physiol. Plant. 82, 67-72.

HAMMERSLEY-STRAW D.R.H. & THORPE T.A. 1988 Use of osmotic inhibition in studies of shoot formation in tobacco callus cultures. Bot. Gaz. 149, 303-310.

HARBAGE J.F., STIMART D.P. & AUER C. 1998 pH affects 1*H*-indole-3-butyric acid uptake but not metabolism during the initiation phase of adventitious root induction in apple microcuttings. J. Am. Soc. Hortic. Sci. 123, 6-10.

HARDING K., BENSON E.E. & CLACHER K. 1997 Plant conservation biotechnology: an overview. Agro Food Ind. Hi Tech. 8, 24-29.

HARRAN S. & DICKINSON D.B. 1978 Metabolism of myo-inositol and growth in various sugars of suspension cultured cells. Planta 141, 77-82.

HARRINGTON H.M. & HENKE R.R. 1981 Amino acid transport into cultured tobacco cells. I. Lysine transport. Plant Physiol. 67, 373-378.

HARRIS R.E. & STEVENSON J.H. 1979 Virus elimination and rapid propagation of grapes *in vitro*. Comb. Proc. Int. Plant Prop. Soc. 29, 95-108.

HARVAIS G. 1982 An improved medium for growing the orchid *Cypripedium reginae* axenically. Can. J. Bot. 60, 2547-2555.

HELGESON J.P., UPPER C.D. & HABERLACH G.T. 1972 Medium and tissue sugar concentrations during cytokinin-controlled growth of tobacco callus tissues. pp. 484-492 in Carr D.J. (ed.) *Plant Growth Substances 1970.* Springer Verlag, Berlin, Heidelberg, New York.

HELLER R. & GAUTHERET R.J. 1949 Sur l'emploi de papier filtre sans cendres commes support pour les cultures de tissues végétaux. Compt. Rend. Seance Soc. Biol., Paris 143, 335-337.

HENDERSON W.E. & KINNERSLEY A.M. 1988 Corn starch as an alternative gelling agent for plant tissue culture. Plant Cell Tissue Organ Cult. 15, 17-22.

HESS D., LEIPOLDT G. & ILLG R.D. 1979 Investigations on the lactose induction of β-galactosides activity in callus tissue cultures of *Nemesia strumosa* and *Petunia hybrida*. Z. Pflanzenphysiol. 94, 45-53.

HEW C.S., TING S.K. & CHIA T.F. 1988 Substrate utilization by *Dendrobium* tissues. Bot. Gaz. 149, 153-157.

HILDEBRANDT A.C., RIKER A.J. & DUGGAR B.M. 1946 The influence of the composition of the medium on growth *in vitro* of excised tobacco and sunflower tissue cultures. Am. J. Bot. 33, 591-597.

HILDEBRANDT A.C., WILMAR J.C., JOHNS H. & RIKER A.J. 1963 Growth of edible chlorophyllous plant tissues *in vitro*. Am. J. Bot. 50, 248-254.

HISAJIMA S. & THORPE T.A. 1981 Lactose-adapted cultured cells of Japanese morning-glory. Acta Physiol. Plant. 3, 187-191.

HISAJIMA S. & THORPE T.A. 1985 Lactose metabolism in lactose-adapted cells of Japanese morning-glory. J. Plant Physiol. 118, 145-151.

HISAJIMA S., ARAI Y. & ITO T. 1978 Changes in sugar contents and some enzyme activities during growth of morning-glory callus (In Japanese). J. Jpn. Soc. Starch Sci. 25, 223-228.

HO W.-J. & VASIL I.K. 1983 Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.): Growth and plant regeneration from embryogenic cell suspension cultures. Ann. Bot. 51, 719-726.

HOMÈS J. & VANSVEREN-VAN ESPEN N. 1973 Effets du saccharose et de la lumière sur le développement et la morphologie de protocormes d'Orchides cultivés *in vitro*. Bull. Soc. Roy. Bot. Belg. 106, 89-106.

HOOKER T.S. & THORPE T.A. 1997 Effects of water deficit stress on the developmental growth of excised tomato roots cultured *in vitro*. In Vitro Cell. Dev.-Pl. 33, 245-251.

HORAN I., WALKER S., ROBERTS A.V., MOTTLEY J. & SIMPKINS I. 1995 Micropropagation of roses: the benefits of pruned mother-plantlets at Stage II and a greenhouse environment at stage III. J. Hortic. Sci., 70, 799-806.

HOWARD B.H. & MARKS T.R. 1987 The *in vitro - in vivo* interface. pp. 101-111 in Jackson M.B., Mantell S.H. & Blake J. (eds.) *Advances in the Chemical Manipulation of Plant Tissue Cultures*. Monograph 16, British Plant Growth Regulator Group, Bristol.

HU T.C., ZIAUDDIN A., SIMION E. & KASHA K.J. 1995 Isolated microscopore culture of wheat (*Triticum aestivum* L.) in a defined media. I: Effects of pretreatment, isolation methods, and hormones. In Vitro Cell. Dev. Biol., Plant. 31, 79-83.

HUANG B., BIRD S., KEMBLE R., MIKI B. & KELLER W. 1991 Plant regeneration from microspore-derived embryos of *Brassica napus*: effect of embryo age, culture temperature, osmotic pressure and abscisic acid. In Vitro Cell. Dev. Plant 27, 28-31.

HUSEMANN W., AMINO S., FISCHER K., HERZBECK H. & CALLIS R. 1990 Light dependent growth and differentiation processes in photoautotrophic cell cultures. pp. 373-378 in Nijkamp *et al.* (eds.) 1990 (q.v.).

HYNDMAN S.E., HASEGAWA P.M. & BRESSAN R.A. 1982 The role of sucrose and nitrogen in adventitious root formation on cultured rose shoots. Plant Cell Tissue Organ Cult. 1, 229-238.

ICHI T., KODA T., ASAI I., HATANAKA A. & SEKIYA J. 1986 Effects of gelling agents on *in vitro* culture of plant tissues. Agric. Biol. Chem. 50, 2397-2399.

ICHIMURA K. & ODA M. 1995 Stimulation of root growth in several vegetables by wood pulp extract. J. Jpn Soc. Hortic. Sci. 63 (Suppl. 4), 797-803.

IKEDA-IWAI, M., UMEHARA M., SATOH S. & KAMADA H. 2003 Stress-induced somatic embryogenesis in vegetative tissues of *Arabidopsis thaliana*. Plant J. 34, 107-114.

ILIĆ-GRUBOR K., ATTREE S. M. & FOWKE L. C. 1998 Induction of microspore-derived embryos of *Brassica napus* L. with polyethylene glycol (PEG) as osmoticum in a low sucrose medium Plant Cell Rep. 17, 329-333.

IMAMURA J. &. HARADA H. 1980. Effects of abscisic acid and water stress on the embryo and plantlet formation in anther culture of *Nicotiana tabacum* cv. Samsun. Z. Pflanzenphysiol. 100, 285-289.

IMAMURA J., OKABE E., KYO M. & HARADA H. 1982 Embryogenesis and plantlet formation through direct culture of isolated pollen of *Nicotiana tabacum* cv. Samsum and *Nicotiana rustica* cv. *rustica*. Plant Cell Physiol. 23, 713-716.

INOUE M. & MAEDA E. 1982 Control of organ formation in rice callus using two-step culture method. pp. 183-184 in Fujiwara A. (ed.) 1982 (q.v.).

ISHIHARA A. & KATANO M. 1982 Propagation of apple cultivars and rootstocks by shoot-tip culture. pp. 733-734 in Fujiwara A. (ed.) 1982 (q.v.).

JACOBSON L., COOPER B.R. & VOLZ M.G. 1971 The interaction of pH and aeration in Cl uptake by barley roots. Physiol. Plant. 25, 432-435.

JACQUIOT C. 1951 Action of meso-inositol and of adenine on bud formation in the cambium tissue of *Ulmus campestris* cultivated *in vitro*. Compt. Rend. Acad. Sci. Paris 233, 815-817.

JAIN R.K., DAVEY M.R. COCKING E.C. & WU R. 1997 Carbohydrate and osmotic requirements for high-frequency plant regeneration from protoplast-derived colonies of *indica* and *japonica* rice varieties. J. Exp. Bot. 48, 751-758.

JAY-ALLEMAND C., CAPELLI P. & CORNU D. 1992 Root development of *in vitro* hybrid walnut microcuttings in a vermiculite-containing gelrite medium. Sci. Hortic. 51, 335-342.

JEANNIN G., BRONNER R. & HAHNE G. 1995 Somatic embryogenesis and organogenesis induced on the immature zygotic embryo of sunflower (*Helianthus annus* L.) cultivated *in vitro*: Role of Sugar. Plant Cell Rep. 15, 200-204.

JEFFS R.A. & NORTHCOTE D.H. 1967 The influence of IAA and sugar on the pattern of induced differentiation in plant tissue culture. J. Cell Sci. 2, 77-88.

JEONG B.R., FUJIWARA K. & KOZAI T. 1995 Environmental control and photoautotrophic micropropagation. Hortic. Rev. 17, 123-170.

JOHANSSON L. & ERIKSSON T. 1984 Effects of carbon dioxide in anther cultures. Physiol. Plant 60, 26-30.

JOHNSON J.L. & EMINO E.R. 1979 *In vitro* propagation of *Mammillaria elongata*. HortScience 14, 605-606.

JOHRI B.M. & GUHA S. 1963 *In vitro* development of onion plants from flowers. pp. 215-223 in Maheshwari and Ranga Swamy (eds.) 1963 (q.v.).

JOY IV R.W., PATEL K.R. & THORPE T.A. 1988 Ascorbic acid enhancement of organogenesis in tobacco. Plant Cell Tissue Organ Cult. 13, 219-228.

JOY IV R.W., PATEL K.R. & THORPE T.A. 1988 Ascorbic acid enhancement of organogenesis in tobacco callus. Plant Cell Tissue Organ Cult. 13, 219-228.

JUMIN H.B. 1995 Plant regeneration via somatic embryogenesis in *Citrus* and its relatives. Phytomorphology 45, 1-8.

JUNG P., TANNER W. & WOLTER K. 1972 The fate of myo-inositol in *Fraximus* tissue cultures. Phytochemistry 11, 1655-1659.

KAHANE R. & RANCILLAC M. 1996 Carbohydrates in onion cultured *in vitro*. Acta Bot. Gall. 143, 117-123

KANABUS J., BRESSAN R.A. & CARPITA N.C. 1986 Carbon assimilation in carrot cells in liquid culture. Plant Physiol. 82, 363-368.

KANG K.S., VEEDER G.T., MIRRASOUL P.J., KANEKO T. & COTTRELL W. 1982 Agar-like polysaccharide produced by a *Pseudomonas* species: Production and basic properties. Appl. Environ. Microbiol. 43, 1086-1091.

KAO K.N. & MICHAYLUK M.R. 1975 Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126, 105-110.

KARSAI I., BEDO Z. & HAYES P.M. 1994 Effect of induction medium pH and maltose concentration on *in vitro* androgenesis of hexaploid winter triticale and wheat. Plant Cell Tissue Organ Cult. 39, 49-53.

KARTHA K.K. 1981 Meristem culture and cryopreservation – Methods and applications. pp. 181-211 in Thorpe T.A. (ed.) *Plant Tissue Culture: Methods and Applications in Agriculture.* Academic Press, New York, London, Toronto, Sydney.

KAUL B. & STABA E.J. 1968 *Dioscorea* tissue cultures. 1. Biosynthesis and isolation of diesgenin from *Dioscorea deltoidea* callus and suspension cells. Lloydia 31, 171-179.

KAUL K. & KOCHHAR T.S. 1985 Growth and differentiation of callus cultures of *Pinus*. Plant Cell Rep. 4, 180-183.

KAUL K. & SABHARWAL P.S. 1972 Morphogenetic studies on *Haworthia*: Establishment of tissue culture and control of differentiation. Am. J. Bot. 59, 377-385.

KAUL K. & SABHARWAL P.S. 1975 Morphogenetic studies on *Haworthia*: Effects of inositol on growth and differentiation. Am. J. Bot. 62, 655-659.

KAVI KISHOR P.B. & MEHTA A.R. 1982 Some aspects of carbohydrate metabolism in organ-differentiating tobacco and non-differentiating cotton callus cultures. pp. 143-144 in Fujiwara A. (ed.) 1982 (q.v.).

KAVI KISHOR P.B. & REDDY G.M. 1986 Regeneration of plants from long-term cultures of *Oryza sativa* L. Plant Cell Rep. 5, 391-393.

KETELLAPPER H.J. 1953 The mechanism of the action of indole-3-acetic acid on the water absorption by *Avena* coleoptile sections. Acta Bot. Neerl. 2, 387-444.

KIM K.S., DAVELAAR E. & DE KLERK G.J. (1994) Abscisic acid controls dormancy development and bulb formation in lily plantlets regenerated *in vitro*. Physiol. Plant. 90, 59-64.

KHAN A., CHAUHAN Y.S. & ROBERTS L.W. 1986 *In vitro* studies on xylogenesis in citrus-fruit vesicles. 2. Effect of pH of the nutrient medium on the induction of cytodifferentiation. Plant Sci. 46, 213-216.

KIM Y.-H. & JANICK J. 1989b Somatic embryogenesis and organogenesis in cucumber. HortScience 24, 702.

KIMBALL S.L., BEVERSDORF W.D. & BINGHAM E.T. 1975 Influence of osmotic potential on the growth and development of soybean tissue cultures. Crop Sci. 15, 750-752.

KING P.J. & STREET H.E. 1977 Growth patterns in cell cultures. pp. 307-387 in Street H.E. (ed.) *Plant Tissue and Cell Culture.* Bot. Monographs Vol.11, Blackwell Scientific Public-ations. Oxford, London.

KINNERSLEY A.M. & HENDERSON W.E. 1988 Alternative carbohydrates promote differentiation of plant cells. Plant Cell Tissue Organ Cult. 15, 17-22.

KINNERSLEY A.M. & HENDERSON W.E. 1988 Alternative carbohydrates promote differentiation of plant cells. Plant Cell Tissue Organ Cult. 15, 3-16.

KIRDMANEE C., KITAYA Y. & KOZAI T. 1995. Effects of CO_2 enrichment and supporting material in-vitro on photoautotrophic growth of eucalyptus plantlets in-vitro and ex-vitro In Vitro Cell. Dev. –Pl. 31, 144-149

KIRKHAM M.B. & HOLDER P.L. 1981 Water osmotic and turgor potentials of kinetin-treated callus. HortScience 16, 306-307.

KITAMURA Y., IKENAGA T., OOE Y., HIRAOKA N. & MIZUKAMI H. 1998 Induction of furanocoumarin biosynthesis in *Glehnia littoralis* cell suspension cultures by elicitor treatment. Phytochemistry 48, 113-117.

KLEIN R.M. & MANOS G.E. 1960 Use of metal chelates for plant tissue cultures. Ann. NY. Acad. Sci. 88, 416-425.

KNUDSON L. 1946 A new nutrient solution for the germination of orchid seed. Am. Orchid Soc. Bull. 15, 214-217.

KOCH K.E. 1996 Carbohydrate-modulated gene expression in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 509-540.

KOCHBA J. & BUTTON J. 1974 The stimulation of embryogenesis and embryoid development in habituated ovular callus from the 'Shamouti' orange (*Citrus sinensis*) as affected by tissue age and sucrose concentration. Z. Pflanzenphysiol. 73, 415-421.

KOCHBA J., SPIEGEL-ROY P., SAAD S. & NEUMANN H. 1978 Stimulation of embryogenesis in *Citrus* tissue culture by galactose. Naturwissenschaften 65, 261-262.

KOETJE D.S., GRIMES H.D., WANG Y.-C. & HODGES T.K. 1989 Regeneration of *indica* rice (*Oryza sativa* L.) from primary callus from immature embryos. J. Plant Physiol. 13, 184-190.

KOMOR E., ROTTER M. & TANNER W. 1977 A proton-cotransport system in a higher plant: sucrose transport in *Ricinus communis*. Plant Sci. Lett. 9, 153-162.

KOMOR E., THOM M. & MARETZKI A. 1981 The mechanism of sugar uptake by sugarcane suspension cells. Planta 153, 181-192.

KOOP H.U., WEBER G. & SCHWEIGER H.G. 1983 Individual culture of selected single cells and protoplasts of higher plants in microdroplets of defined media. Z. Pflanzenphysiol. 112, 21-34.

KORDAN H.A. 1959 Proliferation of excised juice vesicles of lemon *in vitro*. Science 129, 779-780.

KOTT L.S. & BEVERSDORF W.D. 1990 Enhanced plant regeneration from microspore-derived embryos of *Brassica napus* by chilling, partial desiccation and age selection. Plant Cell Tissue Organ Cult. 23, 187-194.

KOZAI T. & SEIKIMOTO K. 1988. Effects of the number of air changes per hour of the closed vessel and the photosynthetic photon flux on the carbon dioxide concentration inside the vessel and the growth of strawberry plantlets *in vitro*. Environ. Contr. Biol. 26, 21-29.

KOZAI T., FUJIWARA K. & KITAYA Y. 1995 Modelling, measurement and control in plant tissue culture. Acta Hortic. 393, 63-73.

KOZAI T. 1991a Photoautotrophic micropropagation. In Vitro Cell. Dev. – Pl. 27, 47-51.

KOZAI T. 1991b Micropropagation under photoautotrophic conditions. pp. 447-469 in Debergh P.C. and Zimmerman R.H. (eds.) *Micropropagation. Technology and Application.* Kluwer Academic Publishers, Dordrecht, Boston, London. ISBN 0-7923-0818-2.

KRIEG N.R. & GERHARDT P. 1981 pp. 143-156 in Gerhardt P. Murray R.G.E., Costilow R.N., Nester E.W., Wood W.A., Krieg N.R. and Briggs Phillips G. Manual of Methods for General Bacteriology. Am. Soc. Microbiol. Washington D.C.

KROMER K. & KUKULCZANKA K. 1985 *In vitro* cultures of meristem tips of *Canna indica* L. Acta Hortic. 167, 279-285.

KUMAR A.S., GAMBORG O.L. & NABORS M.W. 1988 Plant regeneration from suspension cultures of *Vigna aconitifolia*. Plant Cell Rep. 7, 138-141.

KUNITAKE H., IMAMIZO H. & MII M. 1993 Somatic embryogenesis and plant regeneration from immature seed-derived calli of rugosa rose (*Rosa rugosa* Thumb). Plant Sci. 90, 187-194.

KUNITAKE H., NAKASHIMA T., MORI, K. & TANAKA, M. 1997 Normalization of asparagus somatic embryogenesis using a maltose-containing medium. J. Plant Physiol. 150, 458-461.

KURAISHI S. & OKUMURA F.S. 1961 A new green-leaf growth stimulating factor, phyllococosine, from coconut milk. Nature 189, 148-149.

KURATA K., IBARAKI Y. & GOTO E. 1991 System for micro-propagation by nutrient mist supply. Trans. ASAE 34, 621-624.

KURITAKE H., NAKASHIMA T., MORI K. & TANAKA M. 1997 Normalization of asparagus somatic embryogenesis using a maltose-containing medium. J. Plant Physiol. 150, 458-461.

KURKDJIAN A., MATHIEU Y. & GUERN J. 1982 Evidence for an action of 2,4-dichlorophenoxyacetic acid on the vacuolar pH of *Acer pseudoplatanus* cells in suspension culture. Plant Sci. Lett. 27, 77-86.

LA RUE C.D. 1949 Cultures of the endosperm of maize. Am. J. Bot. 36, 798.

LAMOTTE C.E. 1960 The effects of tyrosine and other amino acids on the formation of buds in tobacco callus. Dissertation Abstracts 21, 31-32.

LANDRY L.G. & SMYTH D.A. 1988 Characterization of starch produced by suspension cultures of Indica rice (*Oryza sativa* L.). Plant Cell Tissue Organ Cult. 15, 23-32.

LANG A.R.G. 1967 Osmotic coefficients and water potentials of sodium chloride solutions from 0 to 40ºC. Austr. J. Chem. 20, 2017-2023.

LANGFORD P.J. & WAINWRIGHT H. 1987 Effects of sucrose concentration on the photosynthetic ability of rose shoots *in vitro*. Ann. Bot. 60, 633-640.

LANGHANSOVÁ L., KONRÁDOVÁ H. & VANĚK T. 2004 Polyethylene glycol and abscisic acid improve maturation and regeneration of *Panax ginseng* somatic embryos. Plant Cell Rep. 22, 725-730.

LAPEÑA L., PÉREZ-BERMÚDEZ P. & SEGURA J. 1988 Morphogenesis in hypocotyl cultures of *Digitalis obscura*: influence of carbohydrate levels and sources. Plant Science 57, 247-252.

LARKIN P.J., DAVIES P.A. & TANNER G.J. 1988 Nurse culture of low numbers of *Medicago* and *Nicotiana* protoplasts using calcium alginate beads. Plant Sci. 58, 203-210.

LARKIN P.J., DAVIES P.A. & TANNER G.J. 1988. Nurse cultures of low numbers of *Medicago* and *Nicotiana* protoplasts using calcium alginate beads. Plant Sci. 58, 203-210.

LAROSA P.C., HASEGAWA P.M. & BRESSAN R.A. 1981 Initiation of photoautotrophic potato cells. HortScience 16, 433.

LASSOCINSKI W. 1985 Chlorophyll-deficient cacti in tissue cultures. Acta Hortic. 167, 287-293.

LAZZERI P.A., HILDEBRAND D.F., SUNEGA J., WILLIAMS E.G. & COLLINS G.B. 1988 Soybean somatic embryogenesis: interactions between sucrose and auxin. Plant Cell Rep. 7, 517-520.

LEMOS E.E.P. & BLAKE J. 1996 Micropropagation of juvenile and adult *Annona squamosa*. Plant Cell Tissue Organ Cult. 46, 77-79.

LETHAM D.S. 1966 Regulation of cell division in plant tissues. II. A cytokinin in plant extracts: isolation and interaction with other growth regulators. Phytochemistry 5, 269-286.

LETHAM D.S. 1968 A new cytokinin bioassay and the naturally occurring cytokinin complex. pp. 19-31 in Wightman and Setterfield (eds.) Biochemistry and Physiology of Plant Growth Substances. Runge Press, Ottawa.

LETHAM D.S. 1974 Regulators of cell division in plant tissues. XX. The cytokinins of coconut milk. Physiol. Plant. 32, 66-70.

LETHAM D.S. 1982 A compound in coconut milk which actively promoted radish cotyledon expansion, but exhibited negligible activity in tissue culture bioassays for cytokinins, was identified as the 6 oxypurine, 2-(3-methylbut-2-enylamino)-purin-6-one. Plant Sci. Lett. 26, 241-249.

LEVA A.R., BARROSO M. & MURILLO J.M. 1984 La moltiplicazione del melo con la tecnica della micropropagazione. Vriazione del pH in substrati diversi durante la fase di multiplicazione. Riv. Ortoflorofrutti. Ital. 68, 483-492.

LI X.Y. & HUANG F.H.. 1996 Induction of somatic embryogenesis in Loblolly pine (*Pinus taeda* L.). In Vitro Cell. Dev. – Pl. 32, 129-135.

LI X.Y., HUANG F.H., MURPHY J.B. & GBUR E.R. JR. 1998 Polyethylene glycol and maltose enhance somatic embryo maturation in Loblolly pine (*Pinus taeda* L.). In Vitro Cell. Dev. -Pl. 34, 22-26.

LICHTER R. 1981 Anther culture of *Brassica napus* in a liquid culture medium. Z. Pflanzenphysiol. 103, 229-237.

LIMBERG M., CRESS D. & LARK K.G. 1979 Variants of soybean cells which can grow in suspension with maltose as a carbon-energy source. Plant Physiol. 63, 718-721.

LIN M.-L. & STABA E.J. 1961 Peppermint and spearmint tissue cultures. I. Callus formation and submerged culture. Lloydia 24, 139-145.

LINOSSIER L., VEISSEIRE P., CAILLOUX F. & COUDRET A. 1997 Effects of abscisic acid and high concentrations of PEG on Hevea brasiliensis somatic embryos development. Plant Sci. 124, 183-191.

LINSMAIER E.M. & SKOOG F. 1965 Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18, 100-127.

LIPAVSKA H. & VREUGDENHIL D. 1996 Uptake of mannitol from the media by *in vitro* grown plants. Plant Cell Tissue Organ Cult.45, 103-107.

LIPPMANN B. & LIPPMANN G. 1984 Induction of somatic embryos in cotyledonary tissue of soybean, *Glycine max* L. Merr. Plant Cell Rep. 3, 215-218.

LITZ R.E. 1988 Somatic embryogenesis from cultured leaf explants of the tropical tree *Euphoria longan* Stend. J. Plant Physiol. 132, 190-193.

LIU Y.K., SEKI M. & FURUSAKI S. 1999 Plant cell immobilization in loofa sponge using two-way bubble circular system. J. Chem. Eng. Jpn. 32, 8-14.

LLOYD D., ROBERTS A.V. & SHORT K.C. 1988 The induction *in vitro* of adventitious shoots in *Rosa*. Euphytica 37, 31-36.

LLOYD G. & McCOWN B. 1981 Commercially-feasible micropropagation of Mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Int. Plant Prop. Soc. Proc. 30, 421-427.

LLOYD G. & McCOWN B. 1981 Commercially-feasible micropropagation of Mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Int. Plant Prop. Soc. Proc. 30, 421-427.

LOEWUS F.A. & LOEWUS M.W. 1980 Myo-inositol: Biosynthesis and metabolism. pp. 43-76 in Stumpf and Conn (eds.): *The Biochemistry of Plants* 3. Academic Press N.Y. 43-76.

LOEWUS F.A. 1974 The biochemistry of myo-inositol in plants. Recent Adv. Phytochem. 8, 179-207.

LOEWUS F.A., KELLY S. & NEUFELD E.F. 1962 Metabolism of myo-inositol in plants: Conversion to pectin hemicellulose, D-xylose and sugar acids. Proc. Natl. Acad. Sci. USA 48, 421-425.

LOU H. & KAKO S. 1995 Role of high sugar concentrations in inducing somatic embryogenesis from cucumber cotyledons. Sci. Hortic. 64, 11-20.

LOVELL P.H., ILLSLEY A. & MOORE K.G. 1972 The effects of light intensity and sucrose on root formation, photosynthetic ability, and senescence in detached cotyledons of *Sinapis alba* L. and *Raphanus sativus* L. Ann. Bot. 36, 123-134.

LU C., VASIL I.K. & OZIAS-AKINS P. 1982 Somatic embryogenesis in *Zea mays*. Theor. Appl. Genet. 62, 109-112.

LUMSDEN P.J., PRYCE S. & LEIFERT C. 1990 Effect of mineral nutrition on the growth and multiplication of *in vitro* cultured plants. pp. 108-113 in Nijkamp *et al.* (eds.) 1990 (q.v.).

MADHUSUDHAN R., RAS S.R. & RAVISHANKAR G.A. 1995 Osmolarity as a measure of growth of plant cells in suspension cultures. Enz. Microb. Tech. 17, 989-991.

MAPES M.O. & ZAERR J.B. 1981 The effect of the female gametophyte on the growth of cultured Douglas fir embryos. Ann. Bot. 48, 577-582.

MAHESHAWARI P. AND RANGASWAMY N.S. 1963 (eds.). *Plant Tissue and Organ Culture.* Int. Soc. Plant Morphologists, Univ. Delhi, India.

MARETZKI A. & THOM, M. 1978 Characteristics of a galactose-adapted sugarcane cell line grown in suspension culture. Plant Physiol. 61, 544-548.

MARETZKI A., DELA-CRUZ A. & NICKELL L.G. 1971 Extracellular hydrolysis of starch in sugarcane cell suspensions. Plant Physiol. 48, 521-525.

MARETZKI A., THOM A. & NICKELL L.G. 1972 Influence of osmotic potentials on the growth and chemical composition of sugarcane cell cultures. Hawaii Plant Res. 58, 183-199.

MARETZKI A., THOM M. & NICKELL L.G. 1974 Utilisation and metabolism of carbohydrates in cell and callus cultures. pp.

329-361 in Street H.E. (ed.) *Tissue Culture and Plant Science*. Academic Press, London, New York, San Francisco.

MARGARA J. & RANCILLAC M. 1966 Observations préliminaires sur le rôle du milieu nutritif dans l'initiation florale des bourgeons néoformés *in vitro* chez *Cichorium intybus* L. Compt. Rend. Acad. Sci. Paris 263D, 1455-1458.

MARSOLAIS A.A., WILSON D.P.M., TSUJITA M.J. & SENARATNA T. 1991 Somatic embryogenesis and artificial seed production in Zonal (*Pelargonium horterum*) and Regal (*Pelargonium domesticum*) geranium. Can. J. Bot. 69, 1188-1193.

MARTIN S.M. & ROSE D. 1976 Growth of plant cell (*Ipomoea*) suspension cultures at controlled pH levels. Can. J. Bot. 54, 1264-1270.

MARUYAMA E., ISHII K. & KINOSHITA I. 1998 Alginate encapsulation technique and cryogenic procedures for long-term storage of the tropical forest tree *Guazuma crinita* Mart. *in vitro* cultures. Jarq-Jpn. Agr. Res. Q. 32, 301-309.

MATHES M.C., MORSELLI M. & MARVIN J.W. 1973 Use of various carbon sources by isolated maple callus cultures. Plant Cell Physiol. 14, 797-801.

MAUSETH J.D. 1979 Cytokinin-elicited formation of the pith-rib meristem and other effects of growth regulators on the morphogenesis of *Echino cereus* (Cactaceae) seedling shoot apical meristems. Am. J. Bot. 66, 446-451.

McCANCE R.A. & WIDDOWSON E.M. 1940 *The Chemical Composition of Foods*. British M.R.C. Spec. Rep. Serv. No. 235.

MCGREGOR L.J. & MCHUGHEN A. 1990 The influence of various cultural factors on anther culture of four cultivars of spring wheat (*Triticum aestivum* L.). Can. J. Plant Sci. 70, 183-192.

MEHTA A.R. 1982 Comparative studies on some physiological aspects in organ forming (diploid as well as haploid) tobacco and non-organ forming cotton callus tissues. pp. 125-126 in Fujiwara A. (ed.) 1982 (q.v.).

MELLOR F.C. & STACE-SMITH R. 1969 Development of excised potato buds in nutrient medium. Can. J. Bot. 47, 1617-1621.

MENON M.K.C. & LAL M. 1972 Influence of sucrose on the differentiation of cells with zygote-like potentialities in a moss. Naturwissenschaften 59, 514.

MERKLE S.A., PARROTT W.A. & FLINN B.S. 1995 Morphogenetic aspects of somatic embryogenesis. pp. 155-205 in Thorpe T.A. (ed.) *In Vitro Embryogenesis in Plants*. Kluwer Acad. Publ., Dordrecht.

MERYL SMITH M. & STONE B.A. 1973 Studies on *Lolium multiflorum* endosperm in tissue culture. 1. Nutrition. Aust. J. Biol. Sci. 26, 123-133.

MILLER C.O. 1961 A kinetin-like compound in maize. Proc. Natl. Acad. Sci. USA 47, 170-174.

MILLER J.H. 1968 Fern gametophytes as experimental material. Bot. Rev. 34, 361-426.

MINOCHA S.C. & HALPERIN W. 1974 Hormones and metabolites which control tracheid differentiation with or without concomitant effects on growth in cultured tuber tissue of *Helianthus tuberosus* L. Planta 116, 319-331.

MITCHELL E.D., JOHNSON B.B. & WHITTLE T. 1980 β-galactosidase activity in cultured cotton cells (*Gossypium hirsutum* L.). A comparison between cells growing on sucrose and lactose. In Vitro 16, 907-912.

MOLNAR S.J. 1988 Nutrient modifications for improved growth of *Brassica nigra* cell suspension cultures. Plant Cell Tissue Organ Cult. 15, 257-267.

MONDAL H., MANDAL R.K. & BISWAS B.B. 1972 RNA polymerase from eukaryotic cells. Isolation and purification of enzymes and factors from chromatin of coconut nuclei. Eur. J. Biochem. 25, 463-470.

MONNIER M. 1976 Culture *in vitro* de l'embryon immature de *Capsella bursa-pastoris* Moench (L.). Rev. Cytol. Biol. Vég. 39, 1-120.

MONNIER M. 1978 Culture of zygotic embryos. pp. 277-286 in Thorpe T. A. (ed.) 1978 *Frontiers of Plant Tissue Culture.* Int. Assoc. for Plant Tissue Culture. Distrib. by The Bookstore, Univ. Calgary, Alberta, T2N 1N4, Canada.

MOREL G. & MULLER J.-F. 1964 *In vitro* culture of the apical meristem of the potato. Compt. Rend. Acad. Sci., Paris 258, 5250-5252.

MOREL G. & WETMORE R.H. 1951 Tissue culture of monocotyledons. Am. J. Bot. 38, 138-140.

MOREL G. 1946 Action de l'acid pantothénique sur la croissance de tissus d'Aubépine cultivés *in vitro*. Compt. Rend. Acad. Sci., Paris 223, 166-168.

MORRIS D.A. 2000 Transmembrane auxin carrier systems – Dynamic regulators of polar auxin transport. Plant Growth Reg. 32, 161-172.

MÜLLER J.F., MISSIONIER C. & CABOCHE M. 1983 Low density growth of cells derived from *Nicotiana* and *Petunia* protoplasts: Influence of the source of protoplasts and comparison of the growth- promoting activity of various auxins. Physiol. Plant. 57, 35-41.

MURASHIGE T. & NAKANO R.T. 1968 The light requirement for shoot initiation in tobacco callus culture. Am. J. Bot. 55, 710.

MURASHIGE T. & SKOOG F. 1962 A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15, 473-497.

MURASHIGE T. & TUCKER D.P.H. 1969 Growth factor requirements of citrus tissue culture. pp. 1155-1161 in Chapman H. D. (ed.) Proc. 1st Int. Citrus Symp. Vol. 3, Univ. Calif., Riverside Publication.

MURASHIGE T. & TUCKER D.P.H. 1969 Growth factor requirements of citrus tissue culture. pp. 1155-1161 in Chapman H. D. (ed.) Proc. 1st Int. *Citrus* Symp. Vol. 3, Univ. Calif., Riverside Publication.

MURASHIGE T. 1974 Plant propagation through tissue cultures. Annu. Rev. Plant Physiol. 25, 135-166.

MURASHIGE T., SHABDE M.N., HASEGAWA P.N., TAKATORI F.H. & JONES J.B. 1972 Propagation of asparagus through shoot apex culture. I. Nutrient media for formation of plantlets. J. Am. Soc. Hort. Sci. 97, 158-161.

MURASHIGE T., SERPA M. & JONES J.B. 1974 Clonal multiplication of *Gerbera* through tissue culture. HortScience 9, 175-180.

MUTAFTSCHIEV S., COUSSON A. & TRAN THANH VAN K. 1987 Modulation of cell growth and differentiation by pH and oligosaccharides. pp. 29-42 in Jackson M.B., Mantell S.H. & Blake J. (eds.) *Advances in the Chemical Manipulation of Plant Tissue Cultures*. Monograph 16, British Plant Growth Regulator Group, Bristol.

NAIRN B.J. 1988 Significance of gelling agents in a production tissue culture laboratory. Comb. Proc. Int. Plant Prop. Soc. 1987 37, 200-205.

NARAYANASWAMI S. & LARUE C.D. 1955 The morphogenic effects of various physical factors on the gemmae of *Lunaria*. Phytomorph. 5, 99-109.

NEAL C.A. & TOPOLESKI L.D. 1983 Effects of the basal medium on growth of immature tomato embryos *in vitro*. J. Am. Soc. Hortic. Sci. 108, 434-438.

NEGASH A., KRENS F., SCHAART J. & VISSER B. 2001 *In vitro* conservation of enset under slow-growth conditions. Plant Cell Tissue Organ Cult. 66, 107-111.

NERNST W. 1904 Theorie der Reaktiongeschwindigkeit in heterogenen Systemen. Phys. Chem. 47, 52-55.

NESIUS K.K. & FLETCHER J.S. 1973 Carbon dioxide and pH requirement of non-photosynthetic tissue culture cells. Physiol. Plant. 28, 259-263.

NICHOL J.W., SLADE D., VISS P. & STUARD D.A. 1991 Effect of organic acid pretreatment on the regeneration and development (conversion) of whole plants from callus cultures of alfalfa, *Medicago sativa* L. Plant Sci. 79, 181-192.

NICHOL J.W., SLADE D., VISS P. & STUART D.A. 1991 Effect of organic acid pretreatment on the regeneration and development (conversion) of whole plants from callus cultures of alfalfa, *Medicago sativa* L. Plant Sci. 79, 181-192.

NICKELL L.G. & BURKHOLDER P.R. 1950 Atypical growth of plants. II. Growth *in vitro* of virus tumors of *Rumex* in relation to temperature, pH and various sources of nitrogen, carbon and sulfur. Am. J. Bot. 37, 538-547.

NICKELL L.G. & BURKHOLDER P.R. 1950 Atypical growth of plants. II. Growth *in vitro* of virus tumors of *Rumex* in relation to temperature, pH and various sources of nitrogen, carbon and sulfur. Am. J. Bot. 37, 538-547.

NICKELL L.G. & MARETZKI A. 1969 Growth of suspension cultures of sugarcane cells in chemically defined media. Physiol. Plant. 22, 117-125.

NICKELL L.G. & MARETZKI A. 1970 The utilization of sugars and starch as carbon sources by sugarcane cell suspension cultures. Plant Cell Physiol. 11, 183-185.

NIJKAMP H.I.J., VAN DER PLAS I.H.W. & VAN AARTRIJK J. (eds.) *Progress in Plant Cellular and Molecular Biology*. Proc. VIIth Int. Cong. on Plant Tissue and Cell Culture. Amsterdam, The Netherlands. 24-29 June 1990. Kluwer Academic Publishers, Dortrecht, Netherlands.

NITSCH J.P. & NITSCH C. 1956 Auxin-dependent growth of excised *Helianthus* tissues. Am. J. Bot. 43, 839-851.

NITSCH J.P. & NITSCH C. 1965 Néoformation de fleurs *in vitro* chez une espèce de jours courts: *Plumbaga indica* L. Ann. Phys. Vég. 7, 251-256.

NITZSCHE W. & WENZEL G. 1977 *Haploids in Plant Breeding*. Suppl. 8 to Z. Pflanzenzucht. Parey. Berlin and Hamburg.

NOH E.W., MINOCHA S.C. & RIEMENSCHNEIDER D.E. 1988 Adventitious shoot formation from embryonic explants of red pine (*Pinus resinosa*). Physiol. Plant. 74, 119-124.

NORGAARD J.V. 1997 Somatic embryo maturation and plant regeneration in *Abies nordmanniana* LK. Plant Sci. 124, 211-221.

NORSTOG K. & SMITH J. 1963 Culture of small barley embryos on defined media. Science 142, 1655-1656.

NORSTOG K. & SMITH J. 1963 Culture of small barley embryos on defined media. Science 142, 1655-1656.

NORSTOG K. 1965b Induction of apogamy in megagametophytes of *Zamia integrifolia*. Am. J. Bot. 52, 993-999.

NORSTOG K. 1973 New synthetic medium for the culture of premature barley embryos. In Vitro 8, 307-308.

OBATA-SASAMOTO H. & THORPE T.A. 1983 Cell wall invertase activity in cultured tobacco tissues. Plant Cell Tissue Organ Cult. 2, 3-9.

OBATA-SASAMOTO H., BEAUDOIN-EAGAN L. & THORPE T.A. 1984 C-metabolism during callus induction in tobacco. Plant Cell Tissue Organ Cult. 3, 291-299.

OJIMA K. & OHIRA K. 1980 The exudation and characterization of iron-solubilizing compounds in a rice cell suspension culture. Plant Cell Physiol. 21, 1151-1161.

OKA S. & OHYAMA K. 1982 Sugar utilization in mulberry (*Morus alba* L.) bud culture. pp. 67-68 in Fujiwara A. (ed.) 1982 (q.v.).

OWEN H.R., WENGERD D. & MILLER A.R. 1991 Culture medium pH influenced by basal medium, carbohydrate source, gelling agent, activated charcoal, and medium storage method. Plant Cell Rep. 10, 583-586.

OWENS L.D. & WOZNIAK C.A. 1991 Measurement and effects of gel matric potential and expressibility on production of morphogenic callus by cultured sugarbeet leaf discs. Plant Cell Tissue Organ Cult. 26, 127-133.

PAMPLIN E.J. & CHAPMAN J.M. 1975 Sucrose suppression of chlorophyll synthesis in tissue cultures. J. Exp. Bot. 26, 212-220.

PARFITT D.E., ALMEHDI A.A. & BLOKSBERG L.N. 1988 Use of organic buffers in plant tissue-culture systems. Sci. Hortic. 36, 157-163.

PARIS D. & DUHAMET L. 1953 Action d'un mélange d'acides aminés et vitamines sur la proliferation des cultures de croun-gall de Scorsonère; comparison avec l'action du lait de coco. Compt. Rend. Acad. Sci. Paris 236, 1690-1692.

PARR D.R. & EDELMAN J. 1975 Release of hydrolytic enzymes from the cell walls of intact and disrupted carrot callus tissue. Planta 127, 111-119.

PASQUA G., MANES F., MONACELLI B., NATALE L. & ANSELMI S. 2002 Effects of the culture medium pH and ion uptake in *in vitro* vegetative organogenesis in thin cell layers of tobacco. Plant Sci. 162, 947-955.

PASQUALETTO P.-L., WERGIN W.P. & ZIMMERMAN R.H. 1988 Changes in structure and elemental composition of vitrified leaves of `Gala' apple *in vitro*. Acta Hortic. 227, 352-357.

PASQUALETTO P.-L., ZIMMERMAN R.H. & FORDHAM I. 1986a Gelling agent and growth regulator effects on shoot vitrification of `Gala' apple *in vitro*. J. Am. Soc. Hortic. Sci. 111, 976-980 & 112, 407.

PASQUALETTO P.-L., ZIMMERMAN R.H. & FORDHAM I. 1988b The influence of cation and gelling agent concentrations on vitrification of apple cultivars *in vitro*. Plant Cell Tissue Organ Cult. 14, 31-40.

PATEL K.R. & BERLYN G.P. 1983 Cytochemical investigations on *multiple* bud formation in tissue cultures of *Pinus* coulteri. Can. J. Bot. 61, 575-585.

PECH J.C. & ROMANI R.J. 1979 Senescence of pear fruit cells cultured in a continuously renewed auxin-deprived medium. Plant Physiol. 64, 814-817.

PHILLIPS D.V. & SMITH A.E. 1974 Soluble carbohydrates in soybean. Can. J. Bot. 52, 2447-2452.

PHILLIPS G.C. & COLLINS G.B. 1979 *In vitro* tissue culture of selected legumes and plant regeneration from callus cultures of red clover. Crop Sci. 19, 59-64.

PHILLIPS G.C. & COLLINS G.B. 1984 Chapter 7 – Red Clover and other forage legumes. pp. 169-210 in Sharp W.R., Evans D.A., Ammirato P.V. & Yamada Y. (eds.) *Handbook of Plant Cell Culture. Vol. 2. Crop Species.* Macmillan Publishing Co., New York, London.

PHILLIPS G.C. & HUBSTENBERGER J.F. 1985 Organogenesis in pepper tissue cultures. Plant Cell Tissue Organ Cult. 4, 261-269.

PICCIONI E. & STANDARDI A. 1995 Encapsulation of micropropagated buds of six woody species. Plant Cell Tissue Organ Cult. 42, 221-226.

PICCIONI E. 1997 Plantlets from encapsulated micropropagated buds of M.26 apple rootstock. Plant Cell Tissue Organ Cult. 47, 255-260.

PIERIK R.L.M. 1988 *In vitro* culture of higher plants as a tool in the propagation of horticultural crops. Acta Hortic. 226, 25-40.

PIERIK R.L.M., SPRENKELS P.A., VAN DER HARST B. & VAN DER MEYS Q.G. 1988 Seed germination and further development of plantlets of *Paphiopedilum ciliolare* Pfitz. *in vitro*. Sci. Hortic. 34, 139-153.

PLIEGO-ALFARO F. 1988 Development of an *in-vitro* rooting bioassay using juvenile-stage stem cuttings of *Persea americana* Mill. J. Hort. Sci. 63,295-301.

POLIKARPOCHKINA R.T., GAMBURG K.Z. & KHAVIN E.E. 1979 Cell-suspension culture of maize (*Zea mays* L.). Z. Pflanzenphysiol. 95, 57-67.

POLLARD J.K., SHANTZ E.M. & STEWARD F.C. 1961 Hexitols in coconut milk: their role in the nurture of dividing cells. Plant Physiol. 36, 492-501.

PREECE J.E. & SUTTER E.G. 1991 Acclimatization of micro-propagated plants to the greenhouse and field. pp. 71-93 in Debergh P.C. and Zimmerman R.H. (eds.) *Micropropagation. Technology and Application.* Kluwer Academic Publishers, Dordrecht, Boston, London. ISBN 0-7923-0818-2.

PREIL W. 1991 Application of bioreactors in plant propagation. pp. 425-445 in Debergh P.C. and Zimmerman R.H. (eds.) Micro-propagation. Technology and Application. Kluwer Academic Publishers, Dordrecht, Boston, London. ISBN 0-7923-0818-2.

PRETOVA A. & WILLIAMS E.G. 1986 Direct somatic embryogenesis from immature zygotic embryos of flax (*Linum usitatissimum* L.). J. Plant Physiol. 126, 155-161.

PUA E.C. & CHONG C. 1984 Requirement for sorbitol (D-glucitol) as carbon source for *in vitro* propagation of *Malus robusta* No. 5. Can. J.Bot. 62, 1545-1549.

PUA E.-C., RAGOLSKY E., CHANDLER S.F. & THORPE T.A. 1985a Effect of sodium sulfate on *in vitro* organogenesis of tobacco callus. Plant Cell Tissue Organ Cult. 5, 55-62.

PUA E.-C., RAGOLSKY E. & THORPE T.A. 1985b Retention of shoot regeneration capacity of tobacco callus by Na_2SO_4. Plant Cell Rep. 4, 225-228.

RABE E. 1990 Stress physiology: the functional significance of the accumulation of nitrogen-containing compounds. J. Hortic. Sci. 65, 231-243

RADLEY M. & DEAR E. 1958 Occurrence of gibberellin-like substances in coconut. Nature 182, 1098.

RAGHAVAN V. 1977 Diets and culture media for plant embryos. pp. 361-413 in Recheigl M. Jr. (ed.) 1977 CRC Handbook Series in Nutrition & Food Vol. 4. CRC Press, Baton Rouge, Florida.

RAHMAN M.A. & BLAKE J. 1988 The effects of medium composition and culture conditions on *in vitro* rooting and ex vitro establishment of jackfruit (*Artocarpas heterophyllus* Lam.). Plant Cell Tissue Organ Cult. 13, 189-200.

RAINS D.W. 1989 Plant tissue and protoplast culture: application to stress physiology and biochemistry. In: Jones H.G., Flowers T.J. & Jones M.B. (eds) Plants under stress. Cambridge University Press, London, pp 181-196.

RAINS D.W., VALENTINE R.C. & HOLLAENDER A. (eds.) 1980 *Genetic Engineering of Osmoregulation*. Plenum Press, New York, London.

RAMAGE C.M. & WILLIAMS R.R. 2002 Inorganic nitrogen requirements during shoot organogenesis in tobacco leaf discs. J. Exp. Bot 53, 1437-1443.

RAMMING D.W. 1990 The use of embryo culture in fruit breeding. HortScience 25, 393-398.

RANGA SWAMY N.S. 1963 Studies on culturing seeds of *Orobanche aegyptiaca*. pp. 345-354 in Maheshwari and Ranga Swamy (eds.) 1963 (q.v.).

RANGAN T.S. 1984 Clonal propagation. pp. 68-73 in Vasil I.K. (ed.) *Cell Culture and Somatic Cell Genetics of Plants* Vol. 1. Acad. Press, New York.

RANGAN T.S. 1974 Morphogenic investigations on tissue cultures of *Panicum miliaceum*. Z. Pflanzenphysiol. 72, 456-459.

RANGAN T.S., MURASHIGE T. & BITTERS W.P. 1968 *In vitro* initiation of nucellar embryos in monoembryonic *Citrus*. HortScience 3, 226-227.

RAQUIN C. 1983 Utilization of different sugars as carbon sources for *in vitro* anther culture of petunia. Z. Pflanzenphysiol. 111, Suppl., 453-457.

RAVEN J.A. & SMITH F.A. 1976 Cytoplasmic pH regulation and electrogenic H^+ extrusion. Curr. Adv. Plant Sci. 8, 649-660.

RAVEN J.A. 1986 Biochemical disposal of excess H^+ in growing plants? New Phytol. 104, 175-206.

RAWAL S.K. & MEHTA A.R. 1982 Changes in enzyme activity and isoperoxidases in haploid tobacco callus during organogenesis. Plant Sci. Lett. 24, 67-77.

REDENBAUGH K., VISS P., SLADE D. & FUJII J.A. 1987 Scale-up: artificial seeds. pp. 473-493 in Green C.E, Sommers D.A., Hackett W.P. and Biesboer D.D. (eds.) *Plant Tissue and Cell Culture*. Alan R. Liss, Inc., New York

REIDIBOYM-TALLEUX L., DIEMER F., SOURDIOUX M., CHAPELAIN K. & DE-MARCH G.G. 1999 Improvement of somatic embryogenesis in wild cherry (*Prunus avium*), effect of maltose and ABA supplements. Plant Cell Tissue Organ Cult. 55, 199-209.

REPUNTE V.P., TAYA M. & TONE S. 1995 Preparation of artificial seeds using cell aggregates from horseradish hairy roots encapsulated in alginate gel with paraffin coat. J. Ferment. Bioeng. 79, 83-86.

RIER J.P. & BESLOW D.T. 1967 Sucrose concentration and the differentiation of xylem in callus. Bot. Gaz. 128, 73-77.

RIER J.P. & CHEN P.K. 1964 Pigment induction in plant tissue cultures. Plant Physiol. 39, Suppl.

RIERA M., VALON C., FENZI F., GIRAUDAT J. & LEUNG J. 2005 The genetics of adaptive responses to drought stress: abscisic acid-dependent and abscisic acid-independent signalling components. Physiol. Plant. 123, 111-119.

ROBBINS W.J. & BARTLEY M.A. 1937 Vitamin B, and the growth of excised tomato roots. Science 85, 246-247.

ROBBINS W.J. & SCHMIDT M.B. 1939a Vitamin B6, a growth substance for isolated tomato roots. Proc. Nat. Acad. Sci. Wash. 25, 1-3.

ROBBINS W.J. & SCHMIDT M.B. 1939b Further experiments on excised tomato roots. Am. J. Bot. 26, 149-159.

ROBBINS W.J. 1922 Cultivation of excised root tips and stem tips under sterile conditions. Bot. Gaz. 73, 376-390.

ROBERTS A.V. & SMITH E.F. 1990 The preparation *in vitro* of chrysanthemum for transplantation to soil. (1). Protection of roots by cellulose plugs. Plant Cell Tissue Organ Cult. 21, 129-32.

ROBERTS D.R., SUTTON B.C.S. & FLINN B.S. 1990 Synchronous and high frequency germination of interior spruce somatic embryos following partial drying at high relative humidity. Can. J. Bot. 68, 1086-1090.

ROBERTS-OEHLSCHLAGER, S.L. & DUNWELL J.M. 1990 Barley anther culture – pretreatment on mannitol stimulates production of microspore-derived embryos. Plant Cell Tissue Organ Cult. 20, 235-240.

RODRIGUEZ G. & LORENZO MARTIN J.R. 1987 *In vitro* propagation of Canary Island banana (*Musa acuminata* Colla AAA var. Dwarf Cavendish). Studies of factors affecting culture obtention, preservation and conformity of the plants. Acta Hortic. 212, 577-583.

ROEST S. & BOKELMANN G.S. 1975 Vegetative propagation of *Chrysanthemum morifolium* Ram. *in vitro*. Sci. Hortic. 3, 317-330.

ROMBERGER J.A. & TABOR C.A. 1971 The *Picea abies* shoot apical meristem in culture. I. Agar and autoclaving effects. Am. J. Bot. 58, 131-140.

ROSE D. & MARTIN S.M. 1975 Effect of ammonium on growth of plant cells (*Ipomoea* sp.) in suspension cultures. Can. J. Bot. 53, 1942-1949.

ROSNER A., GRESSEL J. & JAKOB K.M. 1977 Discoordination of ribosomal RNA metabolism during metabolic shifts of *Spirodela* plants. Biochim. Biophys. Acta 474, 386-397.

RUBERY P.H. 1980 The mechanism of transmembrane auxin transport and its relation to the chemiosmotic hypothesis of the

polar transport of auxin. pp. 50-60 in Skoog F. (ed.) 1980 *Plant Growth Substances*. Proc. 10th Int. Cong. Plant Growth Substances, Madison, Wisconsin 1979. Springer Verlag, Berlin, Heidelberg.

RUGINI E., TARINI P. & ROSSODIVITA M.E. 1987 Control of shoot vitrification of almond and olive grown *in vitro*. Acta Hortic. 212, 177-183.

RUSSELL J.A. & McCOWN B.H. 1986 Culture and regeneration of *Populus* leaf protoplasts isolated from non-seedling tissue. Plant Sci. 46, 133-142.

SAALBACH G. & KOBLITZ H. 1978 Attempts to initiate callus formation from barley leaves. Plant Sci. Lett. 13, 165-169.

SACHAR R.C. & IYER R.D. 1959 Effect of auxin, kinetin and gibberellin on the placental tissue of *Opuntia dillenii* Haw. cultured *in vitro*. Phytomorph. 9, 1-3.

SADASIVAN V. 1951 The phosphatases in coconut (*Cocos nucifera*). Arch. Biochem. 30, 159-164.

SAGARE A.P., LEE Y.L., LIN T.C., CHEN C.C. & TSAY H.S. 2000 Cytokinin-induced somatic embryogenesis and plant regeneration in *Corydalis yanhusuo* (Fumariaceae) -a medicinal plant. Plant Sci. 160,139-147.

SAGAWA Y. & KUNISAKI J.T. 1982 Clonal propagation of orchids by tissue culture. pp. 683-684 in Fujiwara A. (ed.) 1982 (q.v.).

SAVAGE A.D., KING J. & GAMBORG O.L. 1979 Recovery of a pantothenate auxotroph from a cell suspension culture of *Datura innoxia* Mill. Plant Sci. Lett. 16, 367-376.

SCHENK R.U. & HILDEBRANDT A.C. 1972 Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50, 199-204.

SCHERER P.A. 1988 Standardization of plant micropropagation by usage of a liquid medium with polyurethane foam plugs or a solidified medium with the gellan gum gelrite instead of agar. Acta Hortic. 226, 107-114.

SCHERER P.A., MULLER E., LIPPERT H. & WOLFF G. 1988 Multielement analysis of agar and gelrite impurities investigated by inductively coupled plasma emission spectrometry as well as physical properties of tissue culture media prepared with agar or gellan gum gelrite. Acta Hortic. 226, 655-658.

SCHOLTEN H.J. & PIERIK R.L.M. 1998 Agar as a gelling agent: differential biological effects *in vitro*. Sci. Hortic. 77, 109-116.

SCHUBERT S. & MATZKE H. 1985 Influence of phytohormones and other effectors on proton extrusion by isolated protoplasts from rape leaves. Physiol. Plant. 64, 285-289.

SCHULLER A. & REUTHER G. 1993 Response of *Abies alba* embryonal-suspensor mass to various carbohydrate treatments. Plant Cell Rep. 12, 199-202.

SCOTT J. & BREEN P. 1988 Sugar uptake by strawberry fruit discs and protoplasts. HortScience 23, 756.

SEARS R.G. & DECKARD E.L. 1982 Tissue culture variability in wheat: callus induction and plant regeneration. Crop Sci. 22, 546-550.

SENARATNA T., MCKERSEE B.D. & BOWLEY S.R. 1989a Desiccation tolerance of alfalfa (*Medicago sativa* L.) somatic embryos. Influence of abscisic acid, stress pretreatments and drying rates. Plant Sci. 65, 253-259.

SENARATNA T., McKERSIE B. & ECCLESTONE S. 1989b Germination of desiccated somatic embryos of alfalfa (*Medicago sativa* L.). Plant Physiol. 89, Suppl., 135.

SERP D., CANTANA E., HEINZEN C., VON STOCKAR U. & MARISON I.W. 2000 Characterization of an encapsulation device for the production of monodisperse alginate beads for cell immobilization. Biotechnol. Bioeng. 70, 41-53.

SERRES R.A. 1988 Effects of sucrose and basal medium concentration on *in vitro* rooting of American chestnut. HortScience 23, 739.

SHANTZ E.M. & STEWARD F.C. 1952 Coconut milk factor: the growth-promoting substances in coconut milk. J. Am. Chem. Soc. 74, 6133.

SHANTZ E.M. & STEWARD F.C. 1955 The identification of compound A from coconut milk as 1,3-diphenylurea. J. Am. Chem. Soc. 77, 6351-6353.

SHANTZ E.M. & STEWARD F.C. 1956 The general nature of some nitrogen free growth-promoting substances from *Aesculus* and *Cocos*. Plant Physiol. 30, Suppl., XXXV.

SHANTZ E.M. & STEWARD F.C. 1964 Growth-promoting substances from the environment of the embryo. II. The growth-stimulating complexes of coconut milk, corn and *Aesculus*. pp. 59-75 in Actes du Coll. Int. C.N.R.S. No. 123.

SHARMA A.K., PRASAD R.N. & CHATURVEDI H.C. 1981 Clonal propagation of *Bougainvillea glabra* `Magnifica' through shoot apex culture. Plant Cell Tissue Organ Cult. 1, 33-38.

SHEAT D.E.G., FLETCHER B.H. & STREET H.E. 1959 Studies on the growth of excised roots. VIII. The growth of excised tomato roots supplied with various inorganic sources of nitrogen. New Phytol. 58, 128-141.

SHEPARD J.F. & TOTTEN R.E. 1977 Mesophyll cell protoplasts of potato: isolation, proliferation and plant regeneration. Plant Physiol. 60, 313-316.

SHILLITO R.D., PASZKOWSKI J. & POTRYKUS I. 1983 Agarose plating and a bead type culture technique enable and stimulate development of protoplast-derived colonies in a number of plant species. Plant Cell Rep. 2, 244-247.

SHININGER T.L. 1979 The control of vascular development. Annu. Rev. Plant Physiol. 30, 313-337.

SHOLTO-DOUGLAS J. 1976 *Advanced Guide to Hydroponics.* Pelham Books, London. ISBN 0-7207-08303.

SHORT K.C., WARBURTON J. & ROBERT A.V. 1987 *In vitro* hardening of cultured cauliflower and chrysanthemum plantlets to humidity. Acta Hortic. 212, 329-334.

SHVETSOV S.G. & GAMBURG K.Z. 1981 Uptake of 2,4-D by corn cells in a suspension culture. Dokl. Akad. Nauk S.S.S.R. 257, 765-768.

SIEVERT R.C. & HILDEBRANDT A.C. 1965 Variations within single cell clones of tobacco tissue cultures. Am. J. Bot. 52, 742-750.

SINGHA S. 1982 Influence of agar concentration on *in vitro* shoot proliferation of *Malus* sp. `Almey' and *Pyrus communis* `Seckel'. J. Am. Soc. Hortic. Sci. 107, 657-660.

SKINNER J.C. & STREET H.E. 1954 Studies on the growth of excised roots. II. Observations on the growth of excised groundsel roots. New Phytol. 53, 44-67.

SKIRVIN R.M. 1981 Fruit crops. pp. 51-139 in Conger B.V. (ed.) *Cloning Agricultural Plants via In Vitro Techniques.* CRC Press, Inc., Boca Raton, Florida.

SKIRVIN R.M. & CHU M.C 1979 *In vitro* propagation of 'Forever Yours' rose (*Rosa hybrida*) HortScience 14, 608-610.

SKIRVIN R.M., CHU M.C., MANN M.L., YOUNG H., SULLIVAN J. & FERMANIAN T. 1986 Stability of tissue culture medium pH as a function of autoclaving, time, and cultured plant material. Plant Cell Rep. 5, 292-294.

SMITH C.W. 1967 A study of the growth of excised embryo shoot apices of wheat *in vitro*. Ann. Bot. 31, 593-605.

SMITH D.L. & KRIKORIAN A.D. 1989 Release of somatic embryogenic potential from excised zygotic embryos of carrot and maintenance of polyembryonic cultures in hormone-free medium. Am. J. Bot. 76, 1832-1843.

SMITH D.L. & KRIKORIAN A.D. 1990a Somatic proembryo production from excised wounded zygotic carrot embryos on hormone-free medium; evaluation of the effects of pH, ethylene and activated charcoal. Plant Cell Rep. 9, 34-37.

SMITH D.L. & KRIKORIAN A.D. 1990b pH control of carrot somatic embryogenesis. pp. 449-453 in Nijkamp *et al.* (eds.) 1990 (q.v.).

SMITH E.F., ROBERTS A.V. & MOTTLEY J. 1990a The preparation *in vitro* of chrysanthemum for transplantation to soil. (2) Improved resistance to desiccation conferred by paclobutrazol. Plant Cell Tissue Organ Cult., 21, 133-40.

SMITH E.F., ROBERTS A.V. & MOTTLEY J. 1990b The preparation *in vitro* of chrysanthemum for transplantation to soil. (3) Improved resistance to desiccation conferred by reduced humidity. Plant Cell Tissue Organ Cult. , 21, 141-5.

SMITH F.A. & RAVEN J.A. 1979 Intercellular pH and its regulation. Annu. Rev. Plant Physiol. 30, 289-311.

SMITH M.M. & STONE B.A. 1973 Studies on *Lolium multiflorum* endosperm in tissue culture. I. Nutrition. Aust. J. Biol. 26, 123-133.

SOCZEK U. & HEMPEL M. 1988 The influence of some organic medium compounds on multiplication of gerbera *in vitro*. Acta Hortic. 226, 643-646.

SOPORY S.K., JACOBSEN E. & WENZEL G. 1978 Production of monohaploid embryoids and plantlets in cultured anthers of *Solanum tuberosum*. Plant Sci. Lett. 12, 47-54.

SORVARI S. 1986a The effect of starch gelatinized nutrient media in barley anther cultures. Ann. Agric. Fenn. 25, 127-133.

SORVARI S. 1986b Differentiation of potato discs in barley starch gelatinized nutrient media. Ann. Agric. Fenn. 25, 135-138.

SORVARI S. 1986c Comparison of anther cultures of barley cultivars in barley-starch and agar gelatinized media. Ann. Agric. Fenn. 25, 249-254.

SPANGENBERG G., KOOP H.-U., LICHTER R. & SCHWEIGER H.G. 1986 Microculture of single protoplasts of *Brassica napus*. Physiol. Plant. 66, 1-8.

STAFFORD A. & DAVIES D.R. 1979 The culture of immature pea embryos. Ann. Bot. 44, 315-321.

STASOLLA C. & YEUNG E.C. 1999 Ascorbic acid improves conversion of white spruce somatic embryos. In Vitro Cell. Dev.-Pl. 35, 316-319.

STASOLLA C., KONG L., YEUNG E.C. & THORPE T.A. 2003 Maturation of somatic embryos in conifers: morphogenesis, physiology, biochemistry and molecular biology. In Vitro Cell. Dev. – Pl. 38, 93-105.

STAUDT G. 1984 The effect of myo-inositol on the growth of callus tissue in *Vitis*. J. Plant Physiol. 116, 161-166.

STEFFEN J.D., SACHS R.M. & HACKETT W.P. 1988 Growth and development of reproductive and vegetative tissues of *Bougainvillea* cultured *in vitro* as a function of carbohydrate. Am. J. Bot. 75, 1219-1224.

STEHSEL M.L. & CAPLIN S.M. 1969 Sugars: autoclaving versus sterile filtration and the growth of carrot root tissue. Life Sci. 8, 1255-1259.

STEINER S. & LESTER R.L. 1972 Studies on the diversity of inositol-containing yeast phospholipids: incorporation of 2-deoxyglucose into lipid. J. Bacteriol. 109, 81-88.

STEINER S., SMITH S., WAECHTER C.J. & LESTER R.L. 1969 Isolation and partial characterization of a major inositol-containing yeast phospholipid in baker's yeast, mannosyl-diinositol, diphosphorylceramide. Proc. Natl. Acad. Sci. USA 64, 1042-1048.

STEINITZ B. (1999) Sugar alcohols display nonosmotic roles in regulating morphogenesis and metabolism in plants that do not produce polyols as primary photosynthetic products. J. Plant Physiol. 155, 1-8.

STEWARD F.C. & CAPLIN S.M. 1951 A tissue culture from potato tuber: the synergistic action of 2,4-D and of coconut milk. Science 113, 518-520.

STEWARD F.C. & CAPLIN S.M. 1952 Investigations on growth and metabolism of plant cells. IV. Evidence on the role of the coconut milk factor. Ann. Bot. 16, 491-504.

STEWARD F.C. & MOHAN RAM H.Y. 1961 Determining factors in cell growth: some implications for morphogenesis in plants. Adv. Morph. 1, 189-265.

STEWARD F.C. & RAO K.V.N. 1970 Investigations on the growth and metabolism of cultured explants of *Daucus carota*. III. The range of responses induced in carrot explants by exogenous growth factors and by trace elements. Planta 91, 129-145.

STEWARD F.C. & SHANTZ E.M. 1956 The chemical induction of growth in plant tissue cultures. pp. 165-186 in Wain and Wightman (eds.) 1956 *The Chemistry and Mode of Action of Plant Growth Substances*. Butterworth Scientific Publications Ltd., London.

STEWARD F.C. & SHANTZ E.M. 1959 The chemical regulation of growth: Some substances and extracts which induce growth and morphogenesis. Annu. Rev. Plant Physiol. 10, 379-404.

STEWARD F.C. 1963 Carrots and coconuts: Some investigations on growth. pp. 178-197 in Maheshwari and Ranga Swamy (eds.) 1963 (q.v.).

STEWARD F.C., CAPLIN S.M. & MILLAR F.K. 1952 Investigations on growth and metabolism of plant cells. I. New techniques for the investigation of metabolism, nutrition, and growth in undifferentiated cells. Ann. Bot. 16, 57-77.

STEWARD F.C., MAPES M.O. & AMMIRATO P.V. 1969 Growth and morphogenesis in tissue and free cell cultures. pp. 329-376 in Steward F.C. (ed.) 1969 *Plant Physiology - A Treatise* Vol. VB. Academic Press, New York, London.

STEWARD F.C., MAPES M.O., KENT A.E. & HOLSTEN R.D. 1964 Growth and development of cultured plant cells. Science 143, 20-27.

STEWARD F.C., SHANTZ E.M., POLLARD J.K., MAPES M.O. & MITRA J. 1961 Growth induction in explanted cells and tissues: Metabolic and morphogenetic manifestations. pp. 193-246 in Rudnick D. (ed.) *Synthesis of Molecular and Cellular Structure*. Ronald Press, New York.

STONE O.M. 1963 Factors affecting the growth of carnation plants from shoot apices. Ann. Appl. Biol. 52, 199-209.

STRAUS J. & LA RUE C.D. 1954 Maize endosperm tissue grown *in vitro*. 1. Culture requirements. Am. J. Bot. 41, 687-694.

STREET H.E. & HENSHAW G.G. 1966 Introduction and methods employed in plant tissue culture. pp. 459-532 in Willmer E.N. *Cells and Tissues in Culture – Methods and Physiology*. Vol 3. Academic Press, London, New York].

STREET H.E. & McGREGOR S.M. 1952 The carbohydrate nutrition of tomato roots. III. The effects of external sucrose concentration on the growth and anatomy of excised roots. Ann. Bot. 16, 185-205.

STREET H.E. 1969 Growth in organised and unorganised systems - knowledge gained by culture of organs and tissue explants. pp. 3-224 in Steward F. C. (ed.) 1969. *Plant Physiology - a Treatise*. 5B. Academic Press, New York.

STREET H.E. 1977 Laboratory organization. pp. 11-30 in Street H.E. (ed.) *Plant Tissue and Cell Culture*. Bot. Monographs Vol.11, Blackwell Scientific Public-ations. Oxford, London.

STREET H.E., McGONAGLE M.P. & LOWE J.S. 1951 Observations on the `staling' of White's medium by excised tomato roots. Physiol. Plant. 4, 592-616.

STREET H.E., McGONAGLE M.P. & McGREGOR S.M. 1952 Observations on the 'staling' of White's medium by excised tomato roots. II. Iron availability. Physiol. Plant. 5, 248-276.

STRICKLAND S.G., NICHOL J.W., McCALL C.M. & STUART D.A. 1987 Effect of carbohydrate source on alfalfa embryogenesis. Plant Sci. 48, 113-121.

STUART D.A., STRICKLAND S.G. & NICHOL J.W. 1986 Enhanced somatic embryogenesis using maltose. U.S. Patent No. 4801545 ,

STUART D.A., STRICKLAND S.G. & WALKER K.A. 1987 Bioreactor production of alfalfa somatic embryos. HortScience 22, 800-803.

SUNDERLAND N. & DUNWELL J.M. 1977 Anther and pollen culture. pp. 223-265 in Street H.E. (ed.) *Plant Tissue and Cell Culture.* Bot. Monographs Vol.11, Blackwell Scientific Publications. Oxford, London.

SUTTLE J.C. & HULTSTRAND J.F. 1994 Role of endogenous abscisic acid in potato microtuber dormancy. Plant Physiol. 105, 891-896.

SWEDLUND B. & LOCY R.D. 1988 Somatic embryogenesis and plant regeneration in two-year old cultures of *Zea diploperennis*. Plant Cell Rep. 7, 144-147.

TABER R.P., ZHANG C. & HU W.S. 1998 Kinetics of Douglas-fir (*Pseudotsuga menziesii*) somatic embryo development. Can. J. Bot. 76, 863-871.

TAKAYAMA S. & MISAWA M. 1979 Differentiation in *Lilium* bulb scales grown *in vitro* - effect of various cultural conditions. Physiol. Plant. 46, 184-190.

TAKAYAMA S. & MISAWA M. 1979 Differentiation in *Lilium* bulb scales grown *in vitro* - effect of various cultural conditions. Physiol. Plant. 46, 184-190.

TANAKA M., HIRANO T., GOI M., HIGASHIURA T., SASAHARA H. & MURASAKI K. 1991 Practical application of a novel disposable film culture vessel in micropropagation. Acta Hortic. 300, 77-84.

TANDEAU DE MARSAC N. & PEAUD-LENOEL C. 1972a Cultures photosynthtique de lignes clonales de cellules de tabac. Compt. Rend. Acad. Sci. Paris 274D, 1800-1802.

TANDEAU DE MARSAC N. & PEAUD-LENOEL C. 1972b Exchanges d'oxygène et assimilation de gaz carbonique de tissus de tabac en culture photosynthétique. Compt. Rend. Acad. Sci. Paris 274D, 2310-2313.

TAUTORUS T.E. & DUNSTAN D.I. 1995 Scale-up of embryonic plant suspension cultures in bioreactors. In: S Jain, P Gupta, and R Newton (Eds) Somatic embryogenesis in woody plants Vol. 1. Kluwer Academic Publishers, The Netherlands pp 265-292.

TEISSON C. & ALVARD D. 1999 *In vitro* production of potato microtubers in liquid medium using temporary immersion. Potato Res. 42, 499-504.

TELLE J. & GAUTHERET R.J. 1947 Sur la culture indéfinie des tissus de la racine de jusquiame (*Hyascyamus niger*). Compt. Rend. Acad. Sci. Paris 224, 1653-1654.

TENG W.L. 1997 Regeneration of *Anthurium* adventitious shoots using liquid or raft culture. Plant Cell Tissue Organ Cult. 49, 153-156.

THOM M., MARETZKI A., KOMOR E. & SAKAI W.S. 1981 Nutrient uptake and accumulation by sugarcane cell cultures in relation to the growth cycle. Plant Cell Tissue Organ Cult. 1, 3-14.

THOMPSON M.R. & THORPE T.A. 1981 Mannitol metabolism in cell cultures. Plant Physiol. 67, Suppl., 27.

THOMPSON M.R., DOUGLAS T.J., OBATA-SASAMOTO H. & THORPE T.A. 1986 Mannitol metabolism in cultured plant cells. Physiol. Plant. 67, 365-369.

THORPE T.A & MEIER D.D. 1973 Sucrose metabolism during tobacco callus growth. Phytochemistry 12, 493-497.

THORPE T.A. 1982 Carbohydrate utilization and metabolism. pp. 325-368 in Bonga J.M. & Durzan D.J. (eds.) *Tissue Culture in Forestry*. Martinus Nijhoff/Dr. W. Junk Publishers, The Hague.

THORPE T.A. & LAISHLEY E.J. 1974 Carbohydrate oxidation during *Nicotiana tabacum* callus growth. Phytochemistry 13, 1323-1328.

THORPE T.A. & LAISHLEY E.J. 1973 Glucose oxidation during shoot initiation in tobacco callus cultures. J. Exp. Bot. 24, 1082-1089.

THORPE T.A. & MEIER D.D. 1972 Starch metabolism, repiration and shoot formation in tobacco callus cultures. Physiol. Plant. 27, 365-369.

THORPE T.A. & MEIER D.D. 1973 Effects of gibberellic acid and abscisic acid on shoot formation in tobacco callus cultures. Physiol. Plant. 29, 121-124.

THORPE T.A. & MEIER D.D. 1974 Starch metabolism in shoot-forming tobacco callus. J. Exp. Bot. 25, 288-294.

THORPE T.A. & MEIER D.D. 1975 Effect of gibberellic acid on starch metabolism in tobacco callus cultured under shoot-forming conditions. Phytomorph. 25, 238-245.

THORPE T.A. & MURASHIGE T. 1968a Some histochemical changes underlying shoot initiation in tobacco callus culture. Am. J. Bot. 55, 710.

THORPE T.A. & MURASHIGE T. 1968b Starch accumulation in shoot-forming tobacco callus cultures. Science 160, 421-422.

THORPE T.A. & MURASHIGE T. 1970 Some histochemical changes underlying shoot initiation in tobacco callus cultures. Can. J. Bot. 48, 277-285.

THORPE T.A. 1982 Physiological and biochemical aspects of organogenesis *in vitro*. pp. 121-124 in Fujiwara A. (ed.) 1982 (q.v.).

THORPE T.A., JOY R.W. IV & LEUNG W.M. 1986 Starch turnover in shoot-forming tobacco callus. Physiol. Plant. 66, 58-62.

TIAN H.Q. & RUSSELL S.D. 1998 Culture-induced changes in osmolality of tobacco cell suspensions using four exogenous sugars. Plant Cell Tiss Organ Cult. 55, 9-13

TIBURCIO A.F., GENDY C.A. & TRAN THANH VAN K. 1989 Morphogenesis in tobacco subepidermal cells: putrescine as a marker of root differentiation. Plant Cell Tissue Organ Cult. 19, 43-54.

TIMBERT R., BARBOTIN J.-N., KERSULEC A., BAZINET C. & THOMAS D. 1995 Physico-chemical properties of the encapsulation matrix and germination of carrot somatic embryos. Biotechnol. Bioeng. 46, 573-578.

TOMAR U.K. & GUPTA S.C. 1988 Somatic embryogenesis and organogenesis in callus cultures of a tree legume - *Albizia richardiana* King. Plant Cell Rep. 7, 70-73.

TOR M., TWYFORD C.T., FUNES I., BOCCON-GIBOD J., AINSWORTH C.C. & MANTELL S.H. 1998 Isolation and culture of protoplasts from immature leaves and embryogenic cell suspensions of *Dioscorea* yams: tools for transient gene expression studies. Plant Cell Tissue Organ Cult. 53, 113-125.

TORRES K. & CARLISI J.A. 1986 Shoot and root organogenesis of *Camellia sasanqua*. Plant Cell Rep. 5, 381-384.

TORREY J.G. 1956 Chemical factors limiting lateral root formation in isolated pea roots. Physiol. Plant. 9, 370-388

TRAN THANH VAN K. & TRINH H. 1978 Plant propagation: non-identical and identical copies. pp. 134-158 in Hughes K.W., Henke R. & Constantin M. (eds.) *Propagation of Higher Plants through Tissue Culture*. Technical Inf. Center, U.S. Dept. Energy. Spingfield, Va. CONF-7804111.

TRELEASE S.F. & TRELEASE H.M. 1933 Physiologically balanced culture solutions with stable hydrogen-ion concentration. Science 78, 438-439.

TREMBLAY F.M. & LALONDE M. 1984 Requirements for *in vitro* propagation of seven nitrogen-fixing *Alnus* species. Plant Cell Tissue Organ Cult. 3, 189-199.

TREMBLAY F.M., NESME X. & LALONDE M. 1984 Selection and micropropagation of nodulating and non-nodulating clones of *Alnus crispa* (Ait.) Pursh. Plant Soil 78, 171-179.

TRINDADE H. & PAIS M.S. 1997 *In vitro* studies on *Eucalyptus globulus* rooting ability. In Vitro Cell. Dev.-Pl. 33, 1-5.

TROLINDER N.L. & GOODIN J.R. 1987 Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant Cell Rep. 6, 231-234.
TULECKE W., WEINSTEIN L.H., RUTNER A. & LAURENCOT H.J. 1961 The biochemical composition of coconut water as related to its use in plant tissue culture. Contrib. Boyce Thomps. 21, 115-128.
TURNER S.R. & SINGHA S. 1990 Vitrification of crabapple, pear and geum on gellan gum-solidified culture medium. HortScience 25, 1648-1650.
UMBECK P., JOHNSON G., BARTON K. & SWAIN W. 1987 Genetically transformed cotton (*Gossypium hirsutum* L.) plants. Nat. Biotechnol. 5, 263-266.
VACIN E.F. & WENT F.W. 1949 Some pH changes in nutrient solutions. Bot. Gaz. 110, 605-613.
VAIN P., MCMULLEN M.D. & FINER J.J. 1993 Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Rep. 12, 84-88.
VAN DER KRIEKEN W.M., BRETELER H., VISSER M.H.M. & JORDI W. 1992 Effect of light and riboflavin on indolebutyric acid-induced root formation on apply *in vitro*. Physiol. Plant. 85, 589-594.
VAN HUYSTEE R.B. 1977 Porphyrin metabolism in peanut cells cultured in sucrose containing medium. Acta Hortic. 78, 83-87.
VAN OVERBEEK J. 1942 Water uptake by excised root systems of the tomato due to non-osmotic forces. Am. J. Bot. 29, 677-683.
VAN OVERBEEK J., CONKLIN M.E. & BLAKESLEE A.F. 1941 Factors in coconut milk essential for growth and development of very young *Datura* embryos. Science 94, 350-351.
VAN OVERBEEK J., CONKLIN M.E. & BLAKESLEE A.F. 1942 Cultivation *in vitro* of small *Datura* embryos. Am. J. Bot. 29, 472-477.
VAN OVERBEEK J., SIU R. & HAAGEN-SMIT A.J. 1944 Factors affecting the growth of *Datura* embryos *in vitro*. Am. J. Bot. 31, 219-224.
VAN STADEN J. & DREWES S.E. 1975 Identification of zeatin and zeatin riboside in coconut milk. Physiol. Plant. 34, 106-109.
VAN STADEN J. 1976 The identification of zeatin glucoside from coconut milk. Physiol. Plant. 36, 123-126.
VANDENBELT J.M. 1945 Nutritive value of coconut. Nature 156, 174-175.
VANSVEREN-VAN ESPEN N. 1973 Effets du saccharose sur le contenu en chlorophylles de protocormes de *Cymbidium* Sw. (Orchidaceae) cultivés *in vitro*. Bull. Soc. Roy. Bot. Belg. 106, 107-115.
VASIL I.K. & HILDEBRANDT A.C. 1966a Variations of morphogenetic behaviour in plant tissue cultures. I. *Cichorium endivia*. Am. J. Bot. 53, 860-869.
VASIL I.K. & HILDEBRANDT A.C. 1966b Variations of morphogenetic behaviour in plant tissue cultures. II. *Petroselinum hortense*. Am. J. Bot. 53, 869-874.
VASIL I.K. & HILDEBRANDT A.C. 1966c Growth and chlorophyll production in plant callus tissues grown *in vitro*. Planta 68, 69-82.
VASIL V. & VASIL I.K. 1981a Somatic embryogenesis and plant regeneration from tissue cultures of *Pennisetum americanum*, and *P. americanum* x *P. purpureum* hybrid. Am. J. Bot. 68, 864-872.
VASIL V. & VASIL I.K. 1981b Somatic embryogenesis and plant regeneration from suspension cultures of pearl millet (*Pennisetum americanum*). Ann. Bot. 47, 669-678.
VENVERLOO C. 1976 The formation of adventitious organs. III. A comparison of root and shoot formation on *Nautilocalyx* explants. Z. Pflanzenphysiol. 80, 310-322.
VERMA D.C. & DOUGALL D.K. 1977 Influence of carbohydrates on quantitative aspects of growth and embryo formation in wild carrot suspension cultures. Plant Physiol. 53, 81-85.
VERMA D.C. & DOUGALL D.K. 1979 Biosynthesis of myo-inositol and its role as a precursor of cell-wall polysaccharides in suspension cultures of wild-carrot cells. Planta 146, 55-62.
VERMA D.P.S. & VAN HUYSTEE R.B. 1971 Derivation, characteristics and large scale culture of a cell line from *Arachis hypogaea* L. cotyledons. Exp. Cell Res. 69, 402-408.
VISEUR J. 1987 Micropropagation of pear, *Pyrus communis* L., in a double-phase culture medium. Acta Hortic. 212, 117-124.
VON ARNOLD S. 1987 Effect of sucrose on starch accumulation in, and adventitous bud formation on, embryos of *Picea abies*. Ann. Bot. 59, 15-22.
VREUGDENHIL D., BOOGAARD Y., VISSER, R.G.F. & DE BRUIJN S.M. 1998 Comparison of tuber and shoot formation from *in vitro* cultured potato explants. Plant Cell Tiss Organ Cult. 53, 197-204.
VYSKOT B. & BEZDEK M. 1984 Stabilization of synthetic media for plant tissue culture and cell cultures. Biol. Plant. 26, 132-143.
VYSKOT B. & JARA Z. 1984 Clonal propagation of cacti through axillary buds *in vitro*. J. Hortic. Sci. 59, 449-452.
WALKER D.R. & PARROTT W.A. 2001 Effect of polyethylene glycol and sugar alcohols on soybean somatic embryo germination and conversion. Plant Cell Tissue Organ Cult. 64, 55-62.
WATAD A.A., AHRONI A., ZUKER A., SHEJTMAN H., NISSIM A. & VAINSTEIN A. 1996 Adventitious shoot formation from carnation stem segments: A comparison of different culture procedures. Sci. Hortic. 65, 313-320
WANN S.R., VEAZEY R. L., KAPHAMMER J. 1997 Activated charcoal does not catalyze sucrose hydrolysis in tissue culture media during autoclaving. Plant Cell Tissue Organ Cult. 50, 221-224.
WATAD A.A., KOCHBA M., NISSIM A. & GABA V. 1995 Improvement of *Aconitum napellus* micropropagation by liquid culture on floating membrane rafts. Plant Cell Rep. 14, 345-348.
WATANABE K., TANAKA K., ASADA K. & KASAL Z. 1971 The growth promoting effect of phytic acid on callus tissues of rice seed. Plant Cell Physiol. 12, 161-164.
WEI Z.M., KYO M. & HARADA H. 1986 Callus formation and plant-regeneration through direct culture of isolated pollen of *Hordeum vulgare* cv. Sabarlis. Theor. Appl. Genet.72, 252-255.
WELANDER T. 1977 *In vitro* organogensis in explants from different cultivars of *Begonia hiemalis*. Physiol. Plant. 41, 142-145.
WESTON G.D. & STREET H.E. 1968 Sugar absorption and sucrose inversion by excised tomato roots. Ann. Bot. 32, 521-529.
WETHERELL D.F. 1969 Phytochrome in cultured wild carrot tissue. I. Synthesis. Plant Physiol. 44, 1734-1737.
WETHERELL D.F. 1984 Enhanced adventive embryogenesis resulting from plasmolysis of cultured wild carrot cells. Plant Cell Tissue Organ Cult. 3, 221-227.
WETMORE R.H. & RIER J.P. 1963 Experimental induction of vascular tissues in callus of angiosperms. Am. J. Bot. 50, 418-430.
WHITE M.C., DECKER A.M. & CHANEY R.L. 1981 Metal complexation in xylem fluid. I. Chemical composition of tomato and soybean stem exudate. Plant Physiol. 67, 292-300.
WHITE P.R. 1932 Influence of some environmental conditions on the growth of excised root-tips of wheat seedlings in liquid media. Plant Physiol. 7, 613-628.
WHITE P.R. 1934 Potentially unlimited growth of excised tomato root tips in a liquid medium. Plant Physiol. 9, 585-600.
WHITE P.R. 1937 Survival of isolated tomato roots at sub-optimal and supra-optimal temperatures. Plant Physiol. 12, 771-776.
WHITE P.R. 1943a *A Handbook of Plant Tissue Culture*. The Jacques Catlell Press, Lancaster, Pa.

WHITE P.R. 1943b Nutrient deficiency studies and an improved inorganic nutrient for cultivation of excised tomato roots. Growth 7, 53-65.

WHITE P.R. 1954 *The Cultivation of Animal and Plant Cells*. 1st. edition. Ronald Press, New York.

WHITE P.R. 1963 *The Cultivation of Animal and Plant Cells*. 2nd edition. Ronald Press, New York.

WHITTIER D.P. & STEEVES T.A. 1960 The induction of apogamy in the bracken fern. Can. J. Bot. 38, 925-930.

WILLIAMS R.R., TAJI A.A. & BOLTON J.A. 1984 *In vitro* propagation of *Dampiera diversifolia* and *Prostanthera rotundifolia*. Plant Cell Tissue Organ Cult. 3, 273-281.

WILLIAMS R.R., TAJI A.M. & BOLTON J.A. 1985 Specificity and interaction among auxins, light and pH in rooting of Australian woody species *in vitro*. HortScience 20, 1052-1053.

WILSON K.S. & CUTTER V.M. 1955 Localization of acid phosphatase in the embryo sac and endosperm of *Cocos nucifera*. Am. J. Bot. 42, 116-119.

WOLF S., KALMAN-ROTEM N., YAKIR D. & ZIV M. 1998 Autotrophic and heterotrophic carbon assimilation of *in vitro* grown potato (*Solanum tuberosum* L.) J. Plant Physiol. 135, 574-580.

WOLTER K.E. & SKOOG F. 1966 Nutritional requirements of Fraxinus callus cultures. Am. J. Bot. 53, 263-269.

WOOD H.N. & BRAUN A.C. 1961 Studies on the regulation of certain essential biosynthetic systems in normal and crown-gall tumor cells. Proc. Natl. Acad. Sci. USA 47, 1907-1913.

WOODWARD S., THOMSON R.J. & NEALE W. 1991 Observations on the propagation of carnivorous plants by *in vitro* culture. Newsl. Int. Plant Prop. Soc. Spring 1991.

WRIGHT K. & NORTHCOTE D.H. 1972 Induced root differentiation in sycamore callus. J. Cell Sci. 11, 319-337.

WRIGHT M.S., KOEHLER S.M., HINCHEE M.A. & CARNES M.G. 1986 Plant regeneration by organogenesis in *Glycine max*. Plant Cell Rep. 5, 150-154.

XIE J., GAO M., CAI Q., CHENG X., SHEN Y. & LIANG Z.. 1995 Improved isolated microspore culture efficiency in medium with maltose and optimized growth regulator combination in japonica rice (*Oryza sativa*). Plant Cell Tissue Organ Cult. 42, 245-250.

XU X., VAN LAMMEREN A.A.M., VERMEER E. & VREUGDENHIL D. 1998 The role of gibberellin, abscisic acid, and sucrose in the regulation of potato tuber formation *in vitro*. Plant Physiol. 117, 575-584.

YATAZAWA M., FURUHASHI K. & SHIMIZU M. 1967 Growth of callus tissue from rice root *in vitro*. Plant Cell Physiol. 8, 363-373.

YOSHIDA F., KOBAYASHI T. & YOSHIDA T. 1973 The mineral nutrition of cultured chlorophyllous cells of tobacco. I. Effects of p salts, p sucrose, Ca, Cl and B in the medium on the yield, friability, chlorophyll contents and mineral absorption of cells. Plant Cell Physiol. 14, 329-339.

YOUNG R.E., HALE A., CAMPER N.D., KEESE R.J. & ADELBERG J.W. 1991 Approaching mechanization of plant micropropagation. Trans. ASAE 34, 328-333

YU S.X. & DORAN P.M. 1994 Oxygen requirements and mass-transfer in hairy-root culture. Biotechnol. Bioeng. 44, 880-887.

ZAGHMOUT O. & TORRES K.C. 1985 The effects of various carbohydrate sources and concentrations on the growth of *Lilium longiflorum* 'Harson' grown *in vitro*. HortScience 20, 660.

ZAMSKI E. & WYSE R.E. 1985 Stereospecificity of glucose carrier in sugar beet suspension cells. Plant Physiol. 78, 291-295.

ZAPATA F.J, KHUSH G.S., CRILL J.P., NEU M.R., ROMERO R.O., TORRIZO L.R. & ALEJAR M. 1983 Rice anther culture at IRRI. pp. 27-86 in *Cell and Tissue Culture Techniques for Cereal Crop Improvement.* Science Press Beijing, China. Gordon and Breach Science Publications, Inc., New York.

ZATYKO J.M. & MOLNAR I. 1986 Adventitious root formation of different fruit species influenced by the pH of medium. p. 29 in Abstracts VI Intl. Cong. Plant Tissue & Cell Culture. Minneapolis, Minn.

ZHANG B., STOLTZ L.P. & SNYDER J.C. 1986 *In vitro* proliferation and *in vivo* establishment of *Euphorbia fulgens*. HortScience 21, 859.

ZHOU T.S. 1995 *In vitro* culture of *Doritaenopsis*: comparison between formation of the hyperhydric protocorm-like-body (PLB) and the normal PLB. Plant Cell Rep. 15, 181-185.

ZHU J.K. (2002) Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 53, 247-273.

ZIMMERMAN R.H. 1979 The laboratory of micropropagation at Cesena, Italy. Comb. Proc. Int. Plant Prop. Soc. 29, 398-400.

ZIV M, 1991. Quality of micropropagated plants - vitrification. In Vitro Cell. Dev. – Pl. 27, 64-69.

ZIV, M. 1989. Enhanced shoot and cormlet proliferation in liquid cultured gladiolus buds by growth retardants. Plant Cell Tissue Organ Cult. 17, 101-110.

ZIV M. 2005 Simple bioreactors for mass propagation of plants. Plant Cell Tissue Organ Cult. 81, 277-285

ZWAR J.A. & BRUCE M.I. 1970 Cytokinins from apple extract and coconut milk. Aust. J. Biol. Sci. 23, 289-297.

ZWAR J.A., KEFFORD N.P., BOTTOMLEY W. & BRUCE M.I. 1963 A comparison of plant cell division inducers from coconut milk and apple fruitlets. Nature 200, 679-680.

Chapter 5
Plant Growth Regulators I: Introduction; Auxins, their Analogues and Inhibitors

1. HORMONES, GROWTH SUBSTANCES AND GROWTH REGULATORS

Some chemicals occurring naturally within plant tissues (*i.e.* endogenously), have a regulatory, rather than a nutritional role in growth and development. These compounds, which are generally active at very low concentrations, are known as *plant hormones* (or *plant growth substances*). Synthetic chemicals with similar physiological activities to plant growth substances, or compounds having an ability to modify plant growth by some other means, for example polyamines, are usually termed *plant growth regulators*. Some of the natural growth substances are prepared synthetically or through fermentation processes and can be purchased from chemical suppliers. When these chemicals have been added to plant tissue culture media, they are termed plant growth regulators in this book, to indicate the fact that they have been applied from outside the tissues (*i.e.* exogenously).

There are several recognised classes of plant growth substance. Until relatively recently only five groups were recognised namely:

- auxins
- cytokinins
- gibberellins
- ethylene
- abscisic acid

Auxins and cytokinins are by far the most important for regulating growth and morphogenesis in plant tissue and organ cultures; in these classes, synthetic regulators have been discovered with a biological activity, which equals or exceeds that of the equivalent natural growth substances.

No chemical alternatives to the natural gibberellins or abscisic acid are available, but some natural gibberellins are extracted from cultured fungi and are available for use as exogenous regulants. However, several classes of chemicals, which are highly effective in blocking the synthesis of gibberellins within the plant, are very effective growth regulators. They are usually termed anti-gibberellins (or growth retardants). These however, can also affect the synthesis of other classes of hormone or growth regulator such as abscisic acid, sterols or brassinosteroids.

Exogenous ethylene can be used as a growth regulant, but being a gas, it is difficult to administer and to control the available concentration, except in tightly sealed vessels, this is also true of other alkynes and alkenes which mimic ethylene action such as acetylene and propylene. However, some chemicals have been invented which are capable of releasing ethylene; effective compounds are taken up into plants as intact molecules, but then break down to release ethylene within the tissue of a plant. One of these ethylene-releasing chemicals – 'ethephon' (2-chlorethanephosphonic acid) is used as a growth regulator for tissue cultures. There are now also some very specific inhibitors both of ethylene biosynthesis and of its action.

In the last decade it has become clear that there are not only five classes of plant hormones but significantly more. Some of these, such as brassinosteroids, jasmonic acid, oligosaccharins and systemin are already relatively well characterised while others - which are known to exist - such as the natural analogues of fusicoccin and the phytotropins - still remain to be identified.

In addition, it has become clear that elicitors and lipochito-oligosaccharides derived from micro-organisms must be considered as plant growth regulators and indeed, it appears that plants possess specific receptor proteins for such substances.

Furthermore, it is now clear that sugars, such as glucose – generally thought of only in terms of their nutritional/energy providing roles – do interact with hormones and/or their transduction chains (see below).

2. AUXINS

Auxins are very widely used in plant tissue culture and usually form an integral part of nutrient media. Auxins promote, mainly in combination with cytokinins, the growth of calli, cell suspensions and organs, and also regulate the direction of morphogenesis. The word auxin has a Greek origin: *auxein* means to enlarge or to grow. At the cellular level, auxins control basic processes such as cell

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division and cell elongation. Since they are capable of initiating cell division they are involved in the formation of meristems giving rise to either unorganised tissue, or defined organs. In organised tissues, auxins are involved in the establishment and maintenance of polarity and in whole plants their most marked effect is the maintenance of apical dominance and mediation of tropisms (summarised in Friml, 2003).

The choice of auxins and the concentration administered depends on:

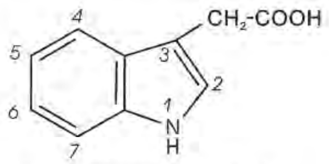
- the type of growth and/or development required;
- the rate of uptake and of transport of the applied auxin to the target tissue;
- the inactivation (oxidation and/or conjugation) of auxin in the medium and within the explant;
- the natural levels and the endogenous synthesis within the explant;
- the sensitivity of the plant tissue to auxin (and other hormones as well);
- the interaction, if any, between applied auxins and the natural endogenous substances.

2.1. NATURALLY OCCURRING AND SYNTHETIC AUXINS

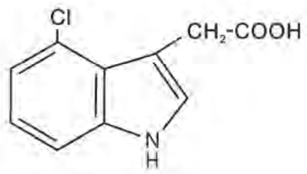
The most commonly detected natural auxin is IAA (indole-3-acetic acid) (1); but endogenous occurrence of 4-chloro-IAA (2) (Engvild, 1985) and of indole-3-butyric acid (IBA) (3) (Ludwig-Müller and Epstein, 1991) have also been demonstrated. Furthermore, the weak auxin phenylacetic acid (PAA) (4) occurs naturally in plants (Okamoto *et al.,* 1967) and there are precursors and metabolites of IAA present in plant tissues, like indole-3-pyruvic acid, tryptamine (Cooney and Nonhebel, 1991) or tryptophol (Rayle and Purves, 1967; Percival *et al.,* 1973).

In addition, the intermediate of agrobacterial IAA biosynthesis, indole-3-acetamide (5), has been detected in plant tissues (Saotome *et al.,* 1993).

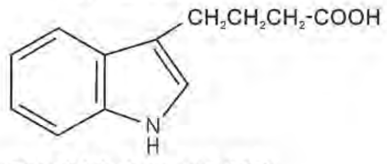
Most of the IAA produced within plants is conjugated to other compounds to form esters, amides or glycosyl esters. The most commonly occurring IAA-conjugates are indole- 3-acetylaspartic acid (IAAsp) (6) and a range of IAA glucose esters (IAAGlu) (7). Conjugation seems to be a mechanism for storing IAA in cells, stabilising the level of free auxin in the plant, and metabolising its excess (Ljung *et al.,* 2002; Normanly *et al.,* 2004). Auxin in conjugated molecules is protected from oxidative breakdown and may be released again through the action of enzymes (see Section 2.2).



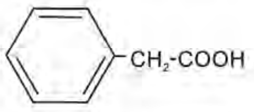
(1) indoleacetic acid (IAA)



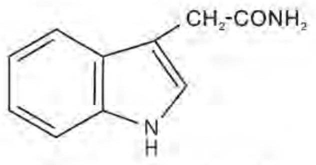
(2) 4Cl-IAA



(3) indolebutyric acid (IBA)



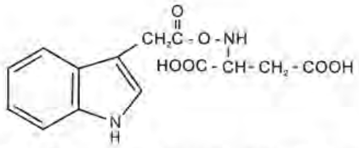
(4) phenylacetic acid (PAA)



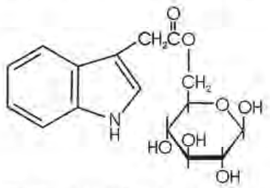
(5) indole-3-acetamide

IAA may be used as an auxin in plant tissue culture media, but it tends to be oxidised in culture media and is rapidly metabolised within plant tissues. However, this characteristic can be useful, because in some plants, callus induced by IAA (together with cytokinins) frequently gives rise to shoots or embryos as its effective concentration becomes diminished. IAA has also been used with other regulants to induce direct morphogenesis (including the rooting of microcuttings), and for meristem and shoot cultures (*e.g.*, of *Bougainvillea*, Chaturvedi *et al.,* 1978; Sharma *et al.,* 1981; *Citrullus*, Barnes, 1979; and

Sinningia, Haramaki, 1971; Grunewaldt, 1977). Also, in this case, decreasing IAA concentration may be important: in apple microcuttings high IAA at the beginning of culture induces root formation but, later on, the growth of roots is promoted by lower IAA concentrations (Guan *et al.,*1997). However, for many purposes, it is necessary or desirable to use one of the many synthetic analogues of IAA. These analogues have different structures but similar biological properties and are also called auxins. The synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D; Table 5.1) and naphthalene acetic acid (NAA; Table 5.1) are not oxidised, but they can also be converted to conjugates with glucose (see below).



(6) 3-indoleacetylaspartic acid (IAAsp)



(7) An example of an IAA glucose ester (IAAGlu)

The synthetic auxins (including selenienylated IAA and 2,4-D) most commonly used in tissue cultures are shown in Table 5.1. Use of these substances in tissue culture is described later in this chapter. Synthetic N-(indole-3-acetyl) amino acids (*e.g.*, conjugates with glycine, alanine, valine, leucine etc.) may serve as an efficient source of IAA in tissue cultures (Magnus *et al.,* 1992) but are only very infrequently used to this purpose.

2.2. PHYSICAL AND CHEMICAL PROPERTIES OF AUXINS

Natural and synthetic auxins are low-molecular-weight, organic substances, containing either an indole or an aromatic ring. They are crystalline and only slightly soluble in water, but readily soluble in organic solvents (ethanol, methanol, acetone, diethyl ether and dimethyl sulphoxide, DMSO) or, as weak acids, in alkaline watery solutions. With the exception of IAA they are stable and persist in the media for tissue cultures. IAA is less stable and is especially sensitive to the light (mainly UV) and to oxidants (see below). Both naturally occurring indolic auxins and their synthetic counterparts, in spite of their different structures, have similar physiological effects. For auxin activity, an aromatic ring is needed at a distance of 0.55 Å from a carboxyl group. Indeed, all active auxins are weak organic acids. The relative degree of activity of individual auxins in different growth processes is very variable. It differs not only from plant to plant, but also from organ to organ, tissue to tissue, cell to cell and, moreover, also with the age and physiological state of the plant (tissue) (Davies, 2004). Probably due to its high instability, IAA is usually less effective than synthetic auxins like 2,4-D or NAA.

3. METABOLISM OF AUXIN

In general, the metabolism of auxin, as well as the metabolism of any other hormone, consists of both biosynthetic and hormone-molecule modifying reactions. Auxin biosynthesis is usually thought to be more intensive in meristematic regions and young growing organs such as rapidly growing leaves, apical buds, root tips, and developing inflorescences.

3.1. NATURAL LEVELS OF IAA

In intact plants, the highest level of IAA occurs predominantly in or near the apex and IAA is transported basipetally; thus, we can observe a decreasing IAA concentration from the top to the bottom. The above is especially true for monocotyledons, whereas in dicotyledons the highest concentration of IAA occurs in the subapical zone, which also grows most rapidly (Law and Davies, 1990). Auxin is also abundant in young leaves, floral organs and developing fruits and seeds. IAA concentrations are high in young, fast growing organs and decline with age and are affected by external factors, e.g. by light (wavelength, intensity and photoperiod). The level is not constant during the day: daily oscillations in IAA concentration in *Chenopodium rubrum* plants have been described. There are no significant changes in constant light, but an endogenous rhythm has been observed in constant darkness. Various diurnal fluctuations were observed in different photoperiodic regimes (Fig 5.1) (Pavlová and Krekule, 1984).

Levels of naturally occurring auxin in explanted tissues are found to depend on the motherplant from which the explants are taken. The age of the motherplant, the conditions under which it has been

growing and the season of the year at which explants are taken, can all be influential (Cassells, 1979). As stated above, the time of the day may also be important. The presence of an associated organised meristem can cause callus to grow more vigorously, or to be capable of organogenesis (Fakhrai *et al.,* 1989). This suggests that meristematic cells are particularly active sites for the biosynthesis and/or the release of natural growth factors favouring cell growth (Clare and Collin, 1974).

3.2. AUXIN BIOSYNTHESIS

Traditionally, the indole amino acid, tryptophan (Trp), which is formed as well as the other aromatic compounds by the shikimic acid pathway, has been thought to be a precursor of the most important native auxin in plants, IAA. However, as described below, Trp-independent pathways have also been considered.

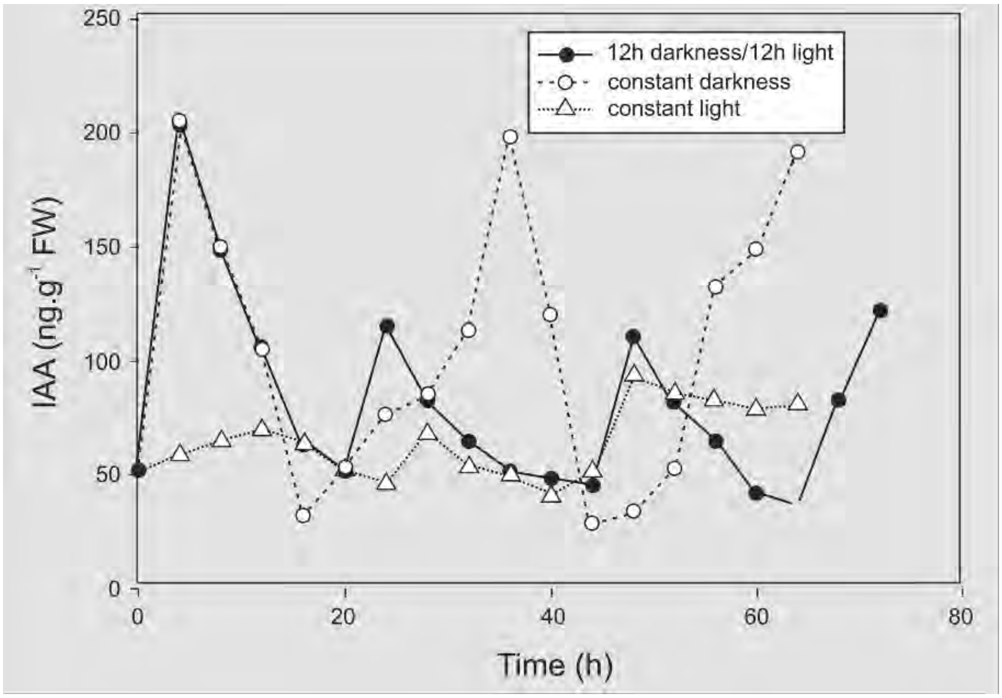


Fig. 5.1 The rhythm in free IAA concentration in *Chenopodium rubrum* plants under three different photoperiodic regimes (Pavlova and Krekule 1984).

There are several possibilities as to how plants may convert Trp into IAA: *via* indole-3-pyruvic acid (the so called "indolepyruvate pathway"), *via* indole-3-acetaldoxime (the "indole-3-acetaldoxime pathway") and *via* tryptamine (the "tryptamine pathway"; Fig 5.2). The most common IAA-biosynthetic pathway in plants appears to be the indolepyruvate one; it begins by the transamination of Trp catalysed by the enzyme tryptophan transaminase (or tryptophan amino transferase) (Forest and Wightman, 1972). Indolepyruvate is then converted into indole-3-acetaldehyde (Gibson *et al.,*1972; Purves and Brown, 1978). IAA can originate from this aldehyde either by dehydrogenation (NAD-dependent indoleacetaldehyde dehydrogenase) or by oxidation (indoleacetaldehyde oxidase) (Wightman and Cohen, 1968; Miyata *et al.,* 1981).

The indole-3-acetaldoxime pathway starts by formation of indole-3-acetaldoxime followed by its conversion to indole-3-acetonitrile and final hydrolysis (nitrilase) to IAA (Thimann and Mahadevan, 1964; Normanly *et al.,* 1997). This pathway is typical for, *e*.g., Brassicaceae and Resedaceae. The tryptamine pathway was reported mainly in Poaceae and involves decarboxylation of Trp into tryptamine (tryptophan decarboxylase) followed by its conversion to indole-3-acetaldehyde. At this point this pathway joins with the indolepyruvate pathway. Since one of the enzymes converting indole-3-acetaldehyde into IAA, *i.e.*, indoleacetaldehyde oxidase, shows sigmoidal

kinetics, its regulatory role for both these pathways has been proposed (Kutacek, 1985).

There is one more IAA biosynthetic pathway starting from Trp: it takes place in some plant pathogenic bacteria and consequently also in plants transformed by *Agrobacterium*. The first step in this pathway is the formation of indole-3-acetamide (Trp monooxygenase) and its subsequent hydrolysis to IAA (indole-3-acetamide hydrolase) (see Klee *et al.,* 1987 for review).

The fact that in labelling experiments the percentage of label in IAA is low in comparison with labelled Trp applied, and that some plants can synthesise IAA even if they are not able to produce Trp (to survive they have to be fed with Trp), led to the recent discovery that IAA can be formed by the non-tryptophan pathway (summarised in Slovin *et al.,* 1999). The probable branch point for IAA and Trp "sub-pathways" lies still in the shikimic acid pathway, and is located upstream from Trp, either at the level of indole-3-glycerol phosphate or indole (Normanly *et al.,* 1993). However, Eckardt in her review summarized evidence suggesting that the Trp-independent pathway might be an artifact (Eckardt 2001).

Hence, all the above mentioned pathways exist in plants, some of them may function in parallel or in some proportion and/or sequence depending on the existing physiological conditions.

3.3. AUXIN CONJUGATION AND DEGRADATION

Usually, the bulk of auxin molecules present in plants are in conjugated forms. Conjugates are compounds in which hormone molecules are bound in a covalent manner with other low-molecular substances, and therefore lose their physiological activity. Hormone-conjugation reactions are mostly reversible and thus may provide a very flexible tool for regulation of endogenous hormone levels.

Auxin is known to form two main classes of conjugates: amides (peptides) and glycosyl esters. Amides originate *via* formation of peptide bonds between auxins (both native and synthetic) and amino acids, namely aspartate, glutamate, alanine, glycine and valine. Glycosyl esters of IAA involve compounds such as various isomers of *o*-indole-3-acetyl-β-D-glucopyranose and indole-3-acetyl-*myo*-inositol, indole-3-acetylglucosyl-rhamnose, etc. (summarised in Cohen and Bandurski, 1982; Slovin *et al.,* 1999, Ljung *et al.,* 2002, Normanly *et al.,* 2004). Some of the auxin conjugates may also serve as transport forms of auxin.

In contrast to conjugation reactions, auxin degradation is an irreversible process. There are two main ways of IAA degradation: oxidative decarboxylation and oxidation without decarboxylation. The oxidative decarboxylation may be catalysed by a non-specific activity traditionally called "IAA-oxidase/peroxidase" and leads to the subsequent formation of degradation products such as indole-3-methanol, indole-3-aldehyde, methylene oxindole and indole-3-carboxylic acid (Hinman and Lang, 1965; reviewed by Reinecke and Bandurski, 1987; Bandurski *et al.,* 1995; Slovin *et al.,* 1999).

Oxidation without decarboxylation involves complex pathways resulting in the formation of oxindole-3-acetic and dioxindole-3-acetic acids and their derivatives. Similar pathways are functional also for degradation of some conjugates, *e.g.*, indole-3-aspartate (see Slovin *et al.,* 1999, for review).

The knowledge on the whole complex of auxin metabolism (including biosynthesis) has been recently summarised in Ljung *et al.*, (2002), Zazimalova and Napier (2003), and Normanly *et al.*, (2004).

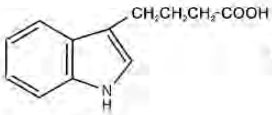
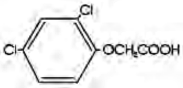
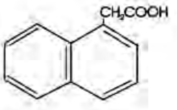
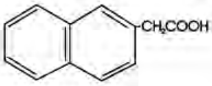
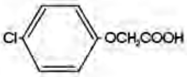
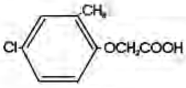
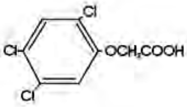
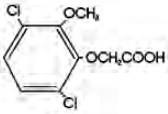
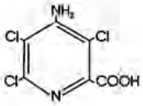
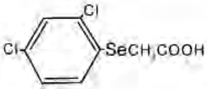
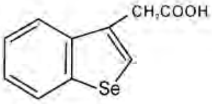
The synthetic auxins, 2,4-D and NAA are often converted, after uptake into plant tissues, to conjugates, mainly glucosyl esters (Barendse *et al.,* 1987, Klems *et al.,* 1998). This reversible conjugation may regulate levels of free active substances.

3.4. EFFECT OF SYNTHETIC AUXINS ON IAA LEVELS

The synthetic auxins, which are added exogenously to control the growth and organisation of cultured tissues, may affect endogenous IAA levels. This can be caused by inhibition of IAA oxidase. Callus cultures of *Arabidopsis thaliana* can be initiated and maintained on a medium containing 2,4-D, but progressively lose their morphogenic capacity the longer they are maintained on it, until after 6-8 months there is no regeneration at all. The loss in regenerative ability was found by Negrutiu *et al.*, (1979) to be closely paralleled by a progressive decline in the activity of peroxidase enzymes through successive transfers of the callus. The decline was reversed each time the callus was removed from the presence of 2,4-D and grown on a shoot-inducing medium. Reduction in peroxidase levels would be expected to result in higher endogenous IAA concentrations, but the decrease in peroxidase enzymes appears in this case to have been associated with a decrease in natural auxin biosynthesis. Nakamura *et al.*, (1998) found a correlation between

decreased rhizogenic activity and the activity of a root-specific peroxidase in tobacco callus. On the other hand, in carrot cell culture, 2,4-D promoted accumulation of tryptophan-derived IAA (Michalczuk *et al.,* 1992).

Table 5.1 Table of synthetic auxins used in tissue cultures

SYNTHETIC AUXINS		
	IBA	indole-3-butyric acid or 1-H-indole-3-butanoic acid *(Note that this compound has also been reported to occur naturally in plants)*
	2,4-D	2,4-dichlorophenoxyacetic acid
	NAA	α-naphthylacetic acid or 1-naphthaleneacetic acid
Less commonly employed compounds include:-		
	NOA	β-naphthoxyacetic acid or 2-naphthyloxyacetic acid
	4-CPA or PCPA	4-chlorophenoxyacetic acid or *para*-chlorophenoxyacetic acid
	MCPA	2-methyl-4-chlorophenoxyacetic acid
	2,4,5-T	2,4,5-trichlorophenoxyacetic acid
	dicamba	3,6-dichloroanisic acid (and its dimethylamine salt, Banvel D)
	picloram	4-amino-3,5,6-trichloropicolinic acid
		2,4-dichlorophenylseleno-acetic acid
		3-(benzo[b]selenienyl) acetic acid

In cultures of wild cherry, endogenous levels of IAA are also considerably reduced by the presence of 2,4-D, although the availability of tryptophan (the precursor of IAA biosynthesis) was increased (Sung, 1979). This suggests that 2,4-D interfered directly with IAA synthesis or hastened IAA conjugation/degradation. 2,4-D inhibition of IAA synthesis has been noted in sycamore suspension cultures (Elliott *et al.,* 1978). Conversely, reducing the external 2,4-D and NAA concentration resulted in a significant increase in internal free IAA concentration in the auxin-dependent and cytokinin-autonomous tobacco cell strain VBI-0 (Zažímalová *et al.,*1995). Maeda and Thorpe (1979) suggested that indole-based synthetic auxins might protect IAA from natural destruction by competing with it for IAA oxidase enzymes.

In carrot hypocotyl explants, neither 2,4-D nor NAA, both of which induced callus formation, had any effect on endogenous IAA concentration. This shows that in this case synthetic auxins induced morphogenesis themselves (Ribnicky *et al.,* 1996). Inhibition of somatic embryos in the globular stage was observed during co-cultivation in growth-regulator-free medium of carrot and *Arabidopsis* somatic embryo cultures. This was probably due to the high intracellular content of 2,4-D in *Arabidopsis* cultures created during preceding cultivation in 2,4-D-containing medium and its release following transfer to growth-regulator-free medium (Meijer *et al.,* 1999).

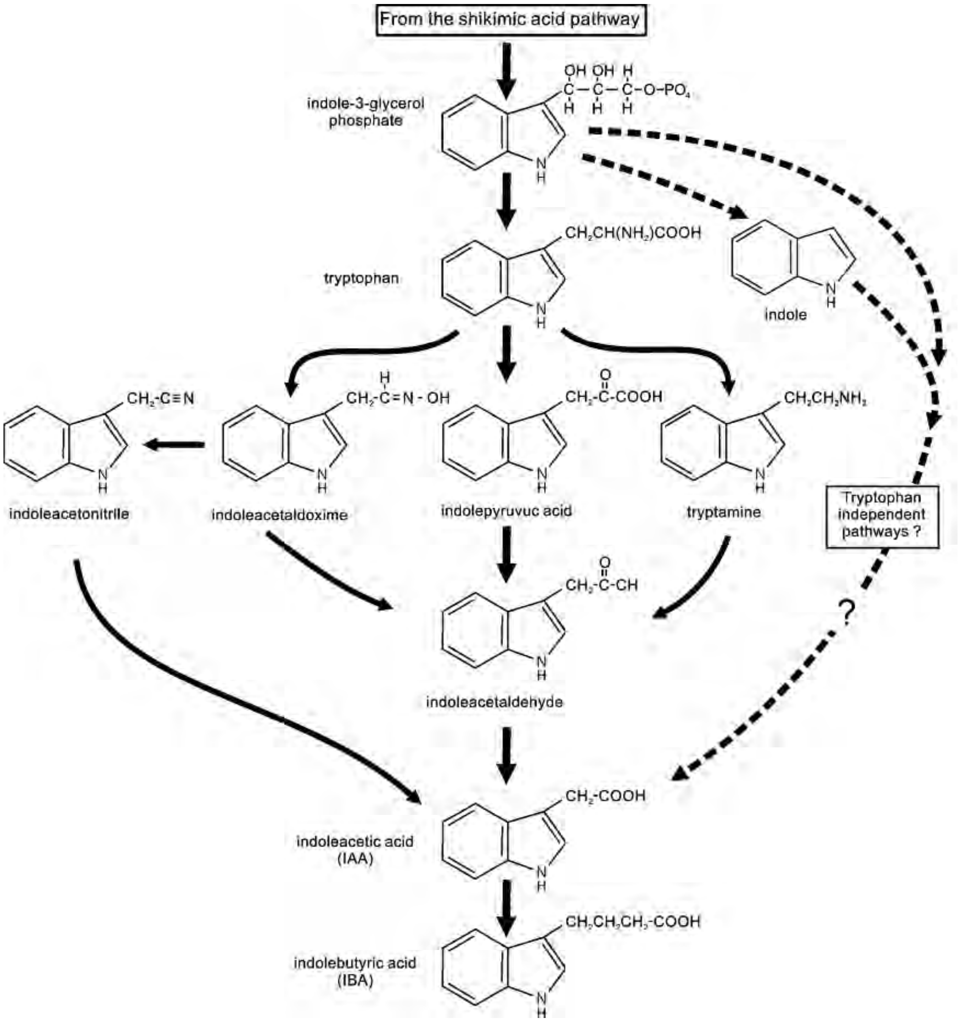


Fig. 5.2 Pathways of IAA biosynthesis.

3.5. STABILITY IN CULTURE MEDIA

IAA and to some extent also IBA are heat labile and decompose during autoclaving. IAA is also unstable in culture media at room temperature. In the dark, there can be more than a ten-fold decrease in concentration over a four-week period in the absence of inocula (Campbell and Sutter, 1986; Nissen and Sutter, 1988). The rate of decrease of IAA is even more rapid in the light and is accelerated by the presence of MS salts (Dunlap *et al.,* 1986). In liquid MS medium incubated at 25°C in a normal 16 h photoperiod, 10 µM (1.75 mg/l) IAA was reduced to less than the limit of detection (0.05 mg/l) in 14 days (Nissen and Sutter, 1988). Because, for oxidative degradation, oxygen is required, in solid media the degradation of IAA is likely to be significantly less. IBA is more stable in solution than IAA: 75% remained unaltered after 30 days in the dark, and 40 % after 30 days in the light. Other auxins such as NAA and 2,4-D are not oxidized (Dunlap *et al.,* 1986), but once absorbed within plant tissues, the rate of either degradation or conjugation of all auxins may be rapid, for they are then not only exposed to physical factors, but are also subjected to enzymatic conversion.

4. TRANSPORT OF AUXINS

Auxins seem to be the only group of plant hormones exhibiting - on the level of the whole plant or its parts - active transport in a polar manner in addition to long-distance movement via vascular tissues (Hopkins, 1995). Auxins appear to be transported long distances extensively through the phloem (but probably not the xylem) of higher plants. The free IAA present in the phloem sap is probably synthesised and exported from the mature leaves. This would accord with Sheldrake's hypothesis (Sheldrake, 1973) that the meristems are net importers rather than synthesisers of IAA in higher plants (reviewed by Baker, 2000) and seems to be in contradiction to the widely accepted idea that meristems are the sites of auxin biosynthesis.

The phenomenon of polar auxin transport was demonstrated first in the late 1920s (Went, 1928). In contrast to the movement of auxin *via* vascular tissues, polar cell-to-cell auxin transport is localised predominantly in parenchyma cells in the sheath surrounding differentiated vascular tissue. The polarity of auxin transport was explained (Rubery and Sheldrake, 1974; Raven, 1975) by the different permeability of opposite parts of cells for dissociated and undissociated molecules of indole-3-acetic acid (IAA^- and IAAH, respectively), and by an asymmetric localisation (basal in the stem cells) of a so-called auxin efflux carrier (translocating the IAA^- anion outside the cell). This idea was summarised by Goldsmith (1977) and named the "chemiosmotic polar diffusion theory". Now, it is believed - on the basis of biochemical, physiological and molecular biological data - that at the level of the individual cell, both passive diffusion and an auxin-uptake (influx) carrier drive auxin translocation into the cell while an auxin efflux carrier drives auxin transport out of the cell. The activity of both carriers, to different extents, can be inhibited by several synthetic compounds, mainly of the phytotropin type, *e.g.* 1-naphthylphthalamic acid (NPA) (8) (reviews by Lomax *et al.,* 1995; Bennett *et al.,* 1998; Morris, 2000). Now several genes are known, coding for both putative carriers and for the NPA-binding site. Typical representatives of these genes, *AUX1* (coding for the putative auxin uptake carrier), *PIN* gene family (coding for the putative auxin efflux carriers, expressed in different tissues and organs) and others have been identified in *Arabidopsis thaliana* (summarised in Galweiler *et al.,* 1998; Morris, 2000; Friml and Palme, 2002; Morris *et al.,* 2004). The effects of potential auxin transport inhibitors on tissue and organ culture are described below.

Delbarre *et al.,* (1996) have studied auxin accumulation at the cell level in detail. Using various radiolabelled auxins, the biochemical properties (kinetic characteristics and specificity) of both carriers (for uptake and efflux) were determined including data on interactions of carriers with potential inhibitors. On the basis of different behaviour of both auxin uptake and efflux carriers towards NAA and 2,4-D, Delbarre *et al.*, (1996) proposed a simple method for measurement of the activity of these carriers: NAA can be used for determination of the efflux carrier, while 2,4-D can serve as a marker of uptake carrier activity. This approach has been used for identification of new auxin transport inhibitors of aryl and aryloxyalkylcarboxylic acid type (Imhoff *et al.,* 2000). Based on studies of auxin transport inhibitors, the hypothesis arose that, unlike the auxin uptake carrier, the auxin efflux carrier has a rather complex structure. It seems to consist of at least two, probably

three components: the carrier itself, the NPA-binding protein and a rapidly turned-over regulatory protein (Morris *et al.,* 1991). Little is known about the mechanisms resulting in proposed asymmetric distribution of this multi-component system. There is some evidence that cytoskeleton (microfilaments in particular) and/or Golgi-mediated protein traffic may be involved in establishment and maintenance of this distribution (reviews by Morris 2000; Friml and Palme, 2002; Morris *et al.,* 2004).

5. MODE OF ACTION OF AUXIN

Plants - like other higher organisms - have to possess intraorganismal communication system(s) working over relatively long distances. As no nervous system is present, the main signalling systems are hormone-dependent (Libbenga and Mennes, 1995). Auxins are a component of such systems. Auxins and cytokinins impact at several levels in many different processes of plant development. At the level of isolated plant cells (grown in cell suspension culture) one can distinguish two main processes apparently controlled by auxins in collaboration with cytokinins, *i*.e., cell cycle and cell division on one hand, and cell elongation on the other. The auxin:cytokinin ratio represents an important signal in the formation of cell phenotype and also in the onset and maintenance of the process of cell division (Stickens *et al.,* 1996; See chapter 10). The ability of auxins (together with cytokinins) to manage key events in plant morphogenesis was documented, among others, by Skoog and Miller's (1957) discovery of the regulation of organogenesis *in vitro* by means of the auxin:cytokinin ratio in culture media (cf. Chapter 6). It has been further supported by recent investigations on the relationships between auxin and cytokinin levels and the morphogenetic response of various plants (*e.g*., Li *et al.,*1994; Leyser *et al.,* 1996; Centeno *et al.,* 1996). However, despite many reports on the physiological action of both individual phytohormones, the molecular mechanisms of their effect(s) on cell expansion, cell division, differentiation, organogenesis, and the mechanisms of their interactions have not yet been elucidated. Nevertheless, the main steps in auxin- (as well as other hormone-) signalling can be generally described as:

1. initial perception of the hormone signal,
2. the signal transduction cascade, and
3. the final physiological response.

5.1. AUXIN SIGNAL PERCEPTION

By analogy to animal systems each target plant cell is presumed to possess receptors, which are able to detect hormonal signals and then to initiate the chain of molecular events leading to the final physiological response. Receptor-like auxin-binding proteins have been identified and characterised by various techniques (traditional ligand-binding studies, photoaffinity labelling and genetic approaches) as recently reviewed by Napier *et al.*, (2002), Zazimalova and Napier (2003), Hagen *et al.*, (2004). There are some candidates for true auxin receptors, especially ZmABP1, *i*.e. ***Zea mays*** **A**uxin-**B**inding **P**rotein **1**, the major auxin-binding protein from maize membranes. This protein exists in the form of a dimer of 22-kDa subunits. It has been purified by several methods and its primary structure was deduced from cDNA clones. Additionally, several other genes encoding this auxin-binding protein (ABP1) have been sequenced from other plants (*Arabidopsis thaliana* and other dicots including tobacco (reviewed in Napier *et al.,* 2002). All homologues share a common primary amino acid sequence containing an N-terminal signal peptide for transit into the endoplasmic reticulum, one glycosylation site and the C-terminal KDEL (Lys-Asp-Glu-Leu) sequence for retention in the lumen of endoplasmic reticulum. The crystal structure of ABP1 and its interaction with auxin was described by Woo *et al.*, (2002). Another membrane-associated ABP, showing specificity very similar to that of the maize ABP1, was detected in tobacco cells cultured *in vitro* (Vreughdenhil *et al.,* 1981 and references therein, Zazimalova *et al.,* 1995). Nevertheless, Jones's (1994) statement: "There is not enough information to label any single ABP as the auxin receptor..." is still valid and the auxin-binding protein story seems to be "curiouser and curiouser" (Timpte, 2001).

5.2. AUXIN SIGNAL TRANSDUCTION PATHWAY(S)

There are several perception/transduction mechanisms known in animal and plant cells (Libbenga and Mennes, 1995; Walden and Lubenow, 1996). There is some indication that transduction of the auxin signal might be mediated by mechanisms based on a plasma membrane-located receptor, a heterotrimeric G protein and phospholipase A_2- or C-catalysed hydrolysis of specific membrane lipids (recent reviews by Millner, 2001; Fujisawa *et al.,*

2001; Scherer, 2002). However, convincing evidence is still missing.

Recent findings suggested the involvement of targeted protein degradation in auxin signalling. This mechanism is based on the regulation of the ubiquitin-conjugating pathway by auxin (Estelle, 1999). Ubiquitin is a small and highly-conserved protein which facilitates protein degradation. It seems to be a rather unexpected way to explain the mode of auxin action. On the other hand, if auxin controls the ubiquitin-mediated degradation of those proteins, which are unique for particular phases of the developmental programme (Fig 5.3) this may be well in agreement with a multifunctional regulatory role of auxin in plant development (Buchanan *et al.,* 2000; Leyser, 2001; Kepinski and Leyser, 2002; Hellman and Estelle, 2002; Dharmasiri and Estelle, 2004).

5.3. AUXIN-REGULATED GENE EXPRESSION

Most auxin-controlled developmental processes involve modulation of gene expression in both positive (up-regulation) and negative (down-regulation) manners (Theologis, 1986). Numerous auxin-responsive genes have been identified (Takahashi *et al.,* 1995; Guilfoyle, 1999). Several families of genes have been identified in a variety of different plants and organs that were rapidly induced after auxin treatment, namely *Aux/IAA, GST* (glutathione-S-transferase), *SAUR* (small-auxin-upregulated RNAs), *ACC* (aminocyclopropane carboxylic acid) synthase, *GH3* genes and many others (summarised in Guilfoyle, 1999).

Fewer auxin-responsive down-regulated genes have been described and these were mostly identified in soybean hypocotyls (Baulcombe and Key, 1980). The main players in the control of transcription by auxin are two families of transcription factors: ARFs (auxin response factors), which can bind to the auxin-response elements within auxin-responsive genes, and Aux/IAA proteins, repressors, the expression of which are auxin-regulated. ARFs can form mixed dimers with Aux/IAAs and in this form they cannot activate expression of relevant genes. At higher auxin concentrations, the ARF-Aux/IAA complexes dissociate and Aux/IAAs are ubiquitinated; ARFs then activate transcription (Ward and Estelle, 2001; Guilfoyle and Hagen, 2001; Kepinski and Leyser, 2002; Tiwari *et al.,* 2004).

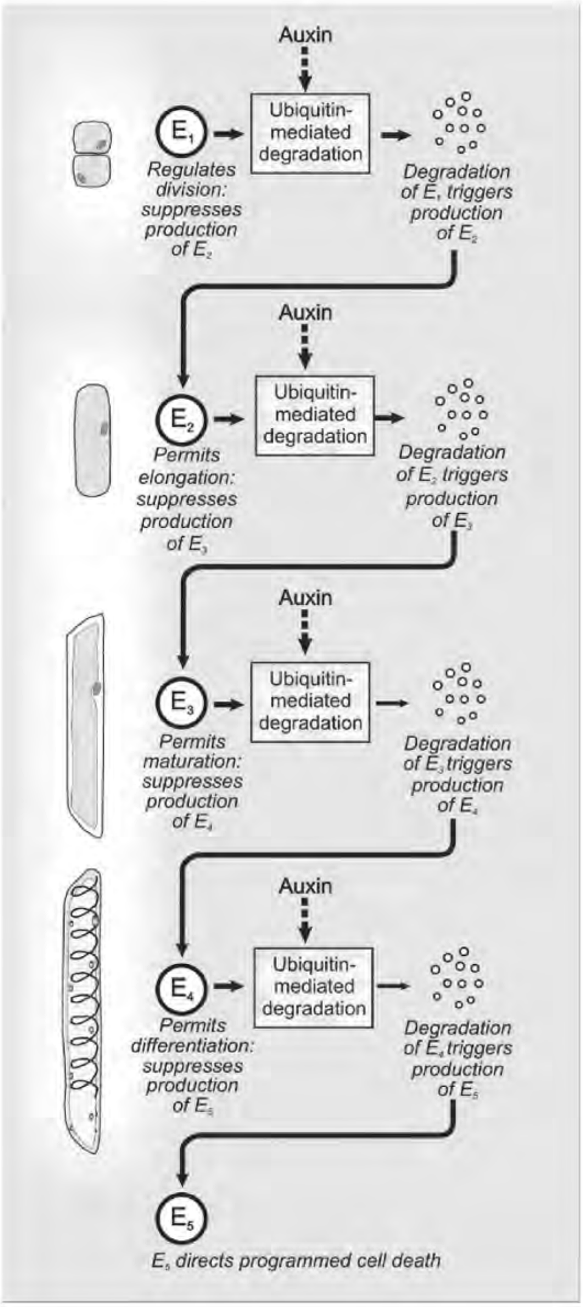


Fig. 5.3 The tentative scheme of auxin-regulated cell development (example: formation of tracheid).
Auxin controls ubiquitin-mediated degradation of hypothetical proteins (E1-E5, i.e. enzymes, repressors, transcription factors, etc.) involved in promotion of particular developmental phases. Degradation of the proteins necessary for one stage results in the commencement of the next one. In this way auxin keeps the development progressing.
(Buchanan *et al.,* 2000 – reproduced with permission).

6. PHYSIOLOGICAL EFFECTS OF AUXINS

In most auxin effects, a bell-shaped concentration/activity curve can be observed. At low concentrations (0.1 - 10 μM) the effect usually increases with concentration, but concentrations higher than 10 μM are often inhibitory. This inhibitory effect is usually due to an increase in ethylene production at higher auxin concentrations (see Chapter 7).

6.1. AT THE CELLULAR LEVEL

A stimulatory effect on cell elongation can be demonstrated in segments of coleoptiles or stems at physiologically significant IAA concentrations. Auxin is in most cases active in the concentration range 0.1 – 10 μM. Activation of cell elongation by auxin is mediated by increased proton efflux ('acid-growth theory', Rayle and Cleland, 1992, Hager *et al.,* 1971) and/or changes in gene expression (see above). Both these theories were later combined into 'dual-sites' hypothesis (Vanderhoef, 1980). This hypothesis can explain the time-course of a typical biphasic auxin-induced elongation curve consisting of short-term (acid growth) and long-term (gene expression) responses (see also Cleland, 2004 for recent review). In nature, an increase in cell division is most obvious in the spring in trees, when young buds produce auxin, which stimulates cell division in the cambium (Sundberg *et al.,* 1991, Funada *et al.,* 2001).

Auxin, together with cytokinins, is also involved in bud initiation and growth. Cell division seems to be regulated by the joint action of auxins and cytokinins (see Chapter 10), each of which appears to influence different phases of the cell cycle. Auxins exert an effect on DNA replication, while cytokinins seem to exert some control over the events leading to mitosis (Jouanneau, 1971,cf Chapter 6). Normal cell divisions require synchrony between the S phase and cell division, suggesting that auxin and cytokinin levels in cultures need to be carefully matched. Auxin starvation resulted in G2-arrest in tobacco cell suspension (Koens *et al.,* 1995). Activation of cell division is also coupled with activation of cdc 2, the main cell cycle regulating kinase (John *et al.,* 1993). Cells are thought not to enter mitosis unless cytokinin is present.

6.2. AT THE TISSUE AND WHOLE PLANT LEVELS

Auxins stimulate differentiation of vascular bundles and, as already discussed, they take part in differentiation of buds and roots (Aloni, 2004). Auxin is gradually canalised by a positive feedback mechanism where increasing conductivity of auxin conducting cells leads to canals of cells efficiently transporting auxin (Sachs, 2000). Polar transport of auxin (see above) is fundamental for the establishment and maintenance of polarity of the plant and its organs. Inhibition of polar auxin transport leads to many abnormalities and in embryos it can lead to death (Liu *et al.,* 1993).

Auxins are known for their ability to promote adventitious root formation. This action is definitely also coupled with stimulation of cell division – increased expression of cyclin B1 and cdc2 was observed well before the first cell division (Hemerly *et al.,* 1993). Early stages of lateral root formation are also regulated by polar auxin transport (Casimiro *et al.,* 2001). IBA is by far the most commonly used auxin to obtain root initiation in conventional cuttings. It has been shown that IBA is readily converted to IAA, but it probably also has an effect on its own (van der Krieken *et al.,* 1992).

Polar transport of auxin is the decisive force of apical dominance (Cline, 1994). Removal of the tip, the main auxin source, or inhibition of auxin transport leads to the outgrowth of axillary buds. Also dominance of fruits is mediated by auxin transport. Uneven distribution of auxin is considered to cause differential growth rates in different sides (upper/lower or irradiated/shaded) of coleoptile or root and their bending in gravitropic or phototropic reactions (Friml, 2003).

7. AUXIN EFFECTS IN TISSUE CULTURE

In tissue culture, depending on other hormones present in the medium, changes in auxin concentrations may change the type of growth, *e.g.*, stimulation of root formation may switch to callus induction etc. In this respect, each tissue culture system is unique, and the effects of different concentrations of auxins and other hormones must be tested for each case individually and only to some extent can the results can be transferred to other cultures.

7.1. INDUCTION OF CALLUS GROWTH

An auxin is generally required for the induction of callus from explants. Applied auxins seem to be capable of fundamentally altering the genetically programmed physiology of whole plant tissues, which had previously determined their differentiated state. Cells, which respond to auxin, revert to a dedifferentiated state and begin to divide. How auxin brings about this reprogramming is understood only to a very limited extent. Lo Schiavo *et al.*, (1989) found that auxins cause DNA to become more methylated than usual and suggested that this might be necessary for the re-programming of differentiated cells. Thus, tissue-specific programmes specifically associated with differentiation would be eradicated by hypermethylation, with perhaps a small fraction of the cells reaching an ultimate state of dedifferentiation in which they become capable of morphogenesis, or embryogenesis (Terzi and Lo Schiavo, 1990). A high rate of DNA methylation was found in the early somatic embryo stage in cultures of *Cucurbita pepo* L (Leljak-Levanic *et al.,* 2004).

Irvine *et al.*, (1983) reported having tested 79 potential regulants for their ability to initiate callus from immature sugarcane leaf tissue. From the effective compounds, 96% had structures known to be associated with auxin activity. The auxin most frequently employed to initiate callus cultures is 2,4-D. However, since cultures maintained on 2,4-D may become genetically variable, some investigators prefer NAA or IAA, or a transfer of callus to a medium containing one of these alternative compounds once it has been initiated by 2,4-D.

For callus induction from broadleaved trees, 2,4-D is generally used at levels between 5 - 15 μM. To induce callus growth from explants of dicotyledonous plants, a cytokinin is usually added to the medium in addition to an auxin. The presence of a cytokinin may not be necessary to obtain callus from explants of monocotyledons and in these plants a somewhat higher auxin concentration, for example 2,4-D in the range 10 - 50 μM is typically used. The combined use of auxin and cytokinin in tissue cultures is considered separately in a later section.

Auxins promote cell dispersion in suspension cultures while cytokinins tend to cause cell aggregation. The relatively high levels of auxin added to liquid media to obtain dispersion will prevent morphogenesis, but might induce embryogenesis if the cells are still competent. Whereas cytokinins tend to promote the formation of chlorophyll in callus and suspension cultures, auxins can be inhibitory. Some reduction of chlorophyll formation in the presence of 2,4-D was noted in callus cultures of pea, tomato and potato by Hildebrandt *et al.*, (1963) and *Oxalis dispar* callus was found to turn green only when the auxin content in the medium was reduced to one tenth of that normally used to promote callus growth (*i.*e., 2,4-D reduced from 5 to 0.5 μM, or NAA from 50 to 5 μM). Compared to callus subcultured to media entirely free of auxin, even a low level of auxin delayed the appearance of chlorophyll and shortened the period over which it accumulated (Sunderland and Wells, 1968). Other workers have made similar observations. Increasing the concentration of IAA led to a progressive reduction in chloroplast development within chicory callus (Wozny *et al.,* 1973), but in other tests IAA or NAA have been reckoned to be less inhibitory to chlorophyll formation than 2,4-D (Davey *et al.,* 1971).

7.2. ORGAN CULTURES

An auxin is almost invariably required to promote the initial growth of meristem and shoot tip explants. A low concentration of auxin is often beneficial in conjunction with high levels of cytokinin at Stage II when shoot multiplication is required, although in some cases cytokinin alone is sufficient. It is important to choose an auxin at a concentration that will promote growth without inducing callus formation.

The induction of rhizogenesis usually requires an adjustment in the levels of auxins and cytokinins. Rhizogenesis is usually achieved by treatment with auxin alone. Also, development of lateral roots is stimulated by auxin as was demonstrated in *Panax ginseng*, where IBA was shown to be more effective than NAA (Kim *et al.,* 2003). Exogenous cytokinins are commonly inhibitory (Reid and Howell, 1995). Auxin-induced root formation is thought to require, or induce, the promotion of polyamine synthesis (Friedman *et al.,* 1985).

Sometimes tissues, organs or strains of cells arise that are able to grow without the addition of any auxin to the medium. They are said to be auxin autonomous or auxin habituated (see Section on habituation below)

7.3. EMBRYOGENESIS

The process of somatic embryogenesis is often initiated in media containing high levels of auxins (especially 2,4-D), but embryos usually do not develop further until the auxin concentration is reduced. Sharp *et al.*, (1980) proposed that auxin

induces an embryogenic determination in a proportion of the cells in callus or suspension cultures but at the same time causes these induced cells to cease further development into embryos. It was suggested that division of the pro-embryogenic cells and their development into embryos is only resumed at lower auxin concentrations. There are, however, many recorded exceptions to this general observation, where somatic embryos are induced even in cultures grown on media devoid of auxins. It is possible that in these instances, embryogenesis has been induced by endogenous auxin, the concentration of which has then been subsequently reduced by metabolism to permit embryo formation. In alfalfa, low 2,4-D concentration gives rise to callus from leaf explants, while higher 2,4-D levels induce formation of embryo-like structures (Fehér *et al.,* 2002). Withdrawal of auxin from the inducing medium is associated with cell death and extracellular acidification in cultures of Norway spruce (Bozhkov *et al.,* 2002).

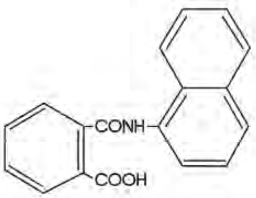
The discovery that embryo formation in carrot can be regulated by pH (see Chapter 4) may imply that at least some of the regulatory effects of auxins on the formation and maintenance of embryogenic cultures can be ascribed to their capacity to reduce intracellular pH. Embryo formation coincides with the withdrawal of auxin and a rise in cellular pH. Formation of cellular pH gradients may be important in the formation of embryogenic cells in alfalfa (Pasternak *et al.,* 2002). However, somatic embryogenesis in carrot has also been found to be induced by an excess of hypochlorite ions (Kiyosue *et al.,* 1989), subjecting tissues to high osmotic potential (0.7 mM sucrose or 0.6 M mannitol in Ishikawa *et al.,* 1988; or 0.3 M NaCl in Kiyosue *et al.,* 1989), and by exposure to heavy metal ions, especially by 0.5-1.0 mM Cd^{2+}, Ni^{2+} and Co^{2+} (Kiyosue *et al.,* 1990). Thus it cannot be assumed that pH is the only controlling factor: a common physiological mechanism by which such different stimuli can induce embryogenesis has yet to be demonstrated. In the induction of somatic embryogenesis from immature cotyledons of *Glycine max*, Lazzeri *et al.*, (1988) discovered a highly significant interaction between the concentration of auxin and sucrose in the medium. The number of embryos obtained was reduced if the concentration ratio of auxin: sucrose was high, or vice versa. In the study of somatic embryogenesis in *Phaseolus vulgaris* induced by 2,4-D, it was shown that there was an inverse relationship between 2,4-D concentration in the medium and endogenous IAA level in the globular structures (Dobrev *et al.,* 2001). Using an immunocytochemical approach to visualize IAA it was shown in sunflower somatic embryos that an endogenous auxin pulse induced by the application of exogenous auxin is needed as one of the first signals inducing embryogenesis (Thomas *et al.,* 2002).

7.4. EXAMPLES OF THE USE OF DIFFERENT SYNTHETIC AUXINS IN TISSUE CULTURES

Together with cytokinins, 2,4-D is used primarily for callus induction and the formation and maintenance of suspension cultures, being replaced by NAA and IBA when morphogenesis is required. NAA and IBA are favoured auxins for shoot culture. 2,4,5 trichlorophenoxyacetic acid (2,4,5-T; Table 5.1) is used only rarely in tissue cultures, and then almost exclusively for the induction of callus and indirect embryogenesis in monocotyledons such as *Avena*, *Oryza*, and *Panicum*. Heyser *et al.*, (1983) found that in *Triticum aestivum*, some varieties produced embryogenic callus with 2,4-D, while others responded only to 2,4,5-T. 3,6-dichloroanisic acid (dicamba; Table 5.1) is often effective in inducing the formation of embryogenic callus in monocotyledons, for example *Dactylis glomerata* (40 μM) (Gray and Conger, 1985); *Musa* (90.5 μM) (Jarret *et al.,* 1985b); and rice (4.5-18.1 μM) (Zimny and Lőrz, 1986). The use of 9.1 μM dicamba permitted the formation of wheat scutellar callus, which produced more somatic embryos in conjunction with 2.6-4.7 μM kinetin, than that induced by the optimum rate of 2,4-D (3.6 μM) (Carman *et al.,* 1988). This is probably because dicamba is metabolised quickly in wheat (Chang and Van den Born, 1971), possibly more quickly than 2,4-D. 4-amino-3,5,6–trichloropicolinic acid (picloram; Table 5.1) is sometimes used to induce and/or maintain callus or suspension cultures of broadleaved trees, or to induce the formation of embryogenic callus, where it may be more effective than 2,4-D (Beyl and Sharma, 1983). The concentration required (*e*.g., 0.06-4.0 μM) is generally less than that necessary for other auxins. Mok and Mok (1977) found that the growth rate and yield of callus from different species and varieties of *Phaseolus*, were greater in the presence of picloram than with 2,4-D. Picloram was active at lower concentrations and over a wider range of genotypes. In only a very few instances has this auxin been used for meristem or single node culture and then at very low concentrations (*e*.g., 0.012-0.4 μM) in combination with a cytokinin.

A comparison of the effectiveness of various synthetic auxins was performed on regeneration of wheat, barley and triticale and it was shown that in barley, dicamba or dicamba + 2,4-D were most effective; in triticale, picloram; and in wheat, dicamba or picloram + 2,4-D (Przetakiewicz *et al.,* 2003). Also seleniated auxins, i.e. the newly described 2,4-dichlorophenylselenoacetic acid (Table 5.1) (Tadino *et al.,* 2003) as well as 3-(benzo[β]selenyl)acetic acid) (BSSA; Table 5.1) induce somatic embryogenesis in *Panax ginseng* (Kevers *et al.,* 2002).

At high concentrations most synthetic auxins are phytotoxic to field-grown broad-leafed plants. 2,4-D, 2,4,5-T, MCPA, dicamba and picloram have been used commercially as selective herbicides. This detrimental effect was reported also for high levels of IAA and is probably due to a dramatic increase in ethylene production. There are many other chemicals with auxin-like activity besides the ones we have listed, which have seldom been used in plant tissue cultures. An unusual auxin 2-chloro-3(2,3-dichlorophenyl) propionitrile (CDPPN) (9), which is related to orthonil (PRB-8) (10), was found by Nemeth (1981) to produce up to 90% more shoots in apple shoot cultures than the same concentration of IBA (5 μM).

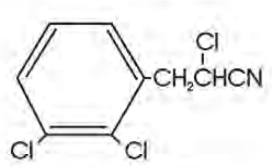


(8) naptalam or NPA
[N-1-naphthylphthalamic acid]

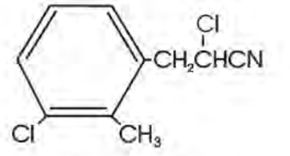
As might also be expected, L-tryptophan can also act as an auxin replacement in some plants. In these cases it may stimulate growth or induce morphogenesis (*e*.g., callus growth of *Nicotiana glauca* x *N. langsdorfii* hybrids - Cheng, 1972; the formation of embryogenic callus in some rice cultivars - Siriwardana and Nabors, 1983).

7.5. MIXTURES OF AUXINS

Some investigators have employed mixtures of many different auxins (*e*.g., Blackmon *et al.,* 1981b), but as the effect of individual compounds can vary in different genotypes, most researchers prefer to use only one, or at most two compounds. However, a mixture of more than one auxin can be particularly effective for root induction and a mixture of a synthetic auxin and IAA has been found by many workers to be more effective than the synthetic compound on its own. A mixture of 2,4-D (or 2,4,5-T) and either IAA or L-tryptophan was found to promote embryogenic callus formation in wheat, pearl millet and some varieties of rice (Nabors *et al.,* 1983). This observation was confirmed by Carman *et al.*, (1988), who found that adding 0.2 mM L-tryptophan to 3.62 μM 2,4-D, consistently enhanced the formation of somatic embryos from scutellum callus of wheat genotypes. There was a decrease in embryo formation when tryptophan was combined with dicamba. As mentioned above, mixtures of auxins are also more effective in inducing regeneration of wheat, barley and triticale (Przetakiewicz *et al.,* 2003).



(9) CDPPN



(10) orthonil

7.6. AUXIN UPTAKE AND METABOLISM IN TISSUE CULTURES

It is not quite correct to talk about uptake, because what we can measure, is in fact accumulation, *i*.e., the amount of a regulator in a tissue, which was taken up from the medium and not yet metabolized. IAA and synthetic auxins such as NAA and 2,4-D are rapidly taken up into cultured tissues from media with a pH less than 5-6. The compounds are subsequently absorbed into cells as whole molecules (via uptake carrier or diffusion, see above), but dissociation then causes them to be retained within the cell, because the plasmalemma is impermeable to auxin anions (Norris and Bukovak, 1972; Raven, 1979; Edwards and Goldsmith, 1980; Minocha and Nissen, 1985; Minocha, 1987). IAA and NAA anions can be exported only by the efflux carrier (see above). Besides uptake through the tissue surface, in cultures using segments, diffusion through the cut surface

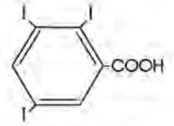
must be taken into account. In apple microcuttings, applied auxin is taken up predominantly via the cut surface and not via the epidermis (Guan and De Klerk, 2000). The rate of uptake of NAA into tobacco pedicel explants was proportional to the concentration in the medium and its presence is necessary for 4 d only (Smulders *et al.,* 1988).

Total 2,4-D uptake (^{14}C-labelled) was found to be higher in easy-to-root juvenile clones of *Sequoiadendron giganteum* than in difficult-to-root mature stem cuttings (Berthon *et al.,* 1991). An unequal distribution of free 2,4-D between apical and basal ends of cuttings was found in both types of shoots; the accumulation was higher in the basal parts. The rate of metabolic conjugation of 2,4-D was higher in the basal part and in the mature clone. 2,4-D uptake and metabolism have also been studied in embryogenic and non-embryogenic maize lines. During the first 24h, uptake was very active in both lines, while after 5 days the embryogenic line contained less 2,4-D. The embryogenic line also metabolised 2,4-D more actively than the non-embryogenic one (Bronsema *et al.,* 1996). Another comparison of 2,4-D uptake, distribution and metabolism was performed with explants of cucumber (*Cucumis sativus* L.) hypocotyls and cotyledons. Cotyledon explants take up more 2,4-D, have a more pronounced basipetal gradient of 2,4-D level and conjugate 2,4-D more actively than hypocotyl explants (Fig. 5.4; Klemš *et al.,* 1998).

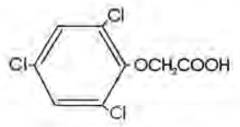
7.7. EFFECT OF AUXIN TRANSPORT INHIBITORS ON SHOOT CULTURES

Polar transport of auxin can be inhibited by NPA (8) or 2,3,5-triiodobenzoic acid (TIBA) (11). In the literature, these compounds are often referred to as 'anti-auxins', but it is more appropiate to use this term only for compounds that compete with auxin for the auxin receptors. 2,4,6-Trichlorophenoxyacetic acid (2,4,6-T) (12) and *p*-chlorophenoxyisobutyric acid (PCIB) (13) are probably genuine anti-auxins. Other compounds with anti-auxin effects are ß-NAA , phenylpropionic acid (14), and 2-(*o*-chlorophenoxy)-2- methylpropionic acid (15).

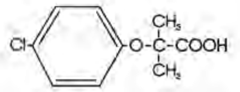
Application of two drops of a 2µM solution of TIBA to the apices of cultured rose shoots had the same effect as manually tipping the shoots, and increased the number of axillary shoots subsequently produced. Adding 6 µM TIBA to the medium for *Rosa hybrida* shoot cultures increased lateral shoot formation during the first two passages (Voyiatzi and Voyiatzi, 1988). TIBA or β-NAA improved adventitious shoot regeneration in sugar beet as well (Zhang *et al.,* 2004).



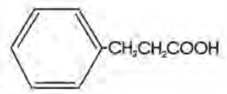
(11) TIBA
[2,3,5-triiodobenzoic acid]



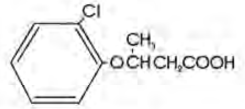
(12) 2,4,6-T
[2,4,6-trichlorophenoxyacetic acid]



(13) PCIB
[*para*-chlorophenoxyisobutyric acid]



(14) phenylpropionic acid



(15) 2-*o*-chlorophenoxy-2-methyl propionic acid

7.8. EFFECTS OF ANTI-AUXINS AND AUXIN TRANSPORT INHIBITORS IN ADVENTIOUS ORGAN FORMATION

Anti-auxins have been reported to promote or modify morphogenesis and in many instances they do appear to have negated the effect of exogenous or endogenous auxin. NPA prevented the growth of tobacco callus when incorporated into the medium at 200 µM, but 2 - 20 µM promoted growth in conjunction with IAA. The compound seemed to reduce auxin activity or enhance that of cytokinin, because callus cultured with 200 µM naptalam plus 12 µM IAA and 2.5 µM kinetin (see Chapter 6)

initiated buds only when NPA was present (Feng and Linck, 1970). TIBA alone improved callus formation and quality in cultures of pepper (Kaparakis and Alderson, 2003).

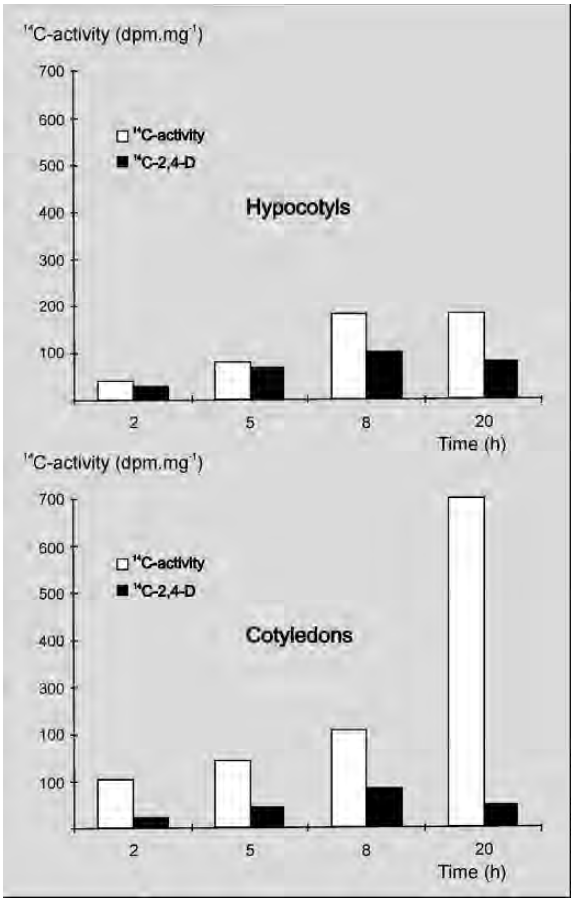


Fig. 5.4 The total ^{14}C-activity (open columns) and the ^{14}C-activity associated with free 2,4-D (solid columns) taken up after 2,5,8 and 20 hours incubation of cucumber cotyledon and hypocotyls explants on induction medium with ^{14}C 2,4-D (Klems *et al.,*1998).

PCIB (13) counteracted the inhibitory effect of 2,4-D on adventitious bud formation on sections of *Chondrilla juncea* roots (Kefford and Caso, 1972) and, together with the cytokinin benzyladenine, it promoted the formation of adventitious shoots from *Solanum melongena* callus, when combinations of IAA and BAP were ineffective. Fiola *et al.*, (1978) thought this might be because PCIB overcame excessive endogenous IAA in the tissue. Similar effects were shown by de Klerk *et al.,*on rooting of apple microcuttings (Fig. 5.5). The work of Cassells (1979) and Cassells *et al.,* (1982) similarly suggested that explants of some species may have a too high level of endogenous auxin for shoot buds to be produced either directly or from associated callus. Placing explants of tomato stems on a medium containing 12 µM cytokinin, zeatin, and 0.5 - 5 µM TIBA but no auxin, caused a marked increase in shoot bud formation, probably because the TIBA prevented endogenous auxin moving to sites of potential shoot bud formation. In *Pelargonium*, callus that formed on MS medium plus zeatin, gave rise to numerous green xylem-containing nodules. These could be induced to develop into shoot buds when TIBA was added to the medium. In the absence of TIBA, the microscopic bud initials of *Pelargonium* seemed to release natural auxin in sufficient concentration to prevent further shoot development.

Callus of *Colocasia esculenta* produced normal shoots when 50 µM TIBA was added to the medium whereas shoots produced with combinations of auxin and cytokinin had an abnormal morphology (Nyman and Arditti, 1984). However, although barley callus developed adventitious shoots on a medium with 2,4,5-T, it produced roots on a medium containing TIBA (Jelaska *et al.,* 1984).

Also, rooting induced by auxins may be influenced by antiauxins and auxin transport inhibitors. Regeneration of roots on tomato hypocotyl cuttings is suppressed by TIBA (Tyburski and Tretyn, 2004). Similarly, NPA inhibited root formation and growth in cultures of oak shoot segments (Vidal *et al.,* 2003).

7.9. EFFECTS OF ANTI-AUXINS AND AUXIN TRANSPORT INHIBITORS ON EMBRYOGENESIS

The effect of auxin transport inhibitors and antiauxins on somatic embryogenesis or embryo growth has been equivocal. In anther cultures of *Hordeum* (Clapham, 1973) and *Zea mays* (Genovesi and Collins, 1982), TIBA was more effective than normal auxins in inducing callus formation and embryogenesis. 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) (12) has also been reported to enhance somatic embryo formation in callus cultures (Newcomb and Wetherell, 1970; Smith and Street, 1974), but Stange (1979) found that PCIB (13) inhibited meristematic activity, and Fujimura and Komamine (1979) discovered that 2,4,6-T and PCIB inhibited embryogenesis in carrot suspensions. They took particular care to wash residual auxin from cell clusters and thought that the stimulatory effects of anti-auxins noted by others, were due to their failure to take this precaution. PCIB is able to inhibit cell proliferation in embryogenic culture of Nordmanns fir and to promote development of embryos (Find *et al.*, 2002),

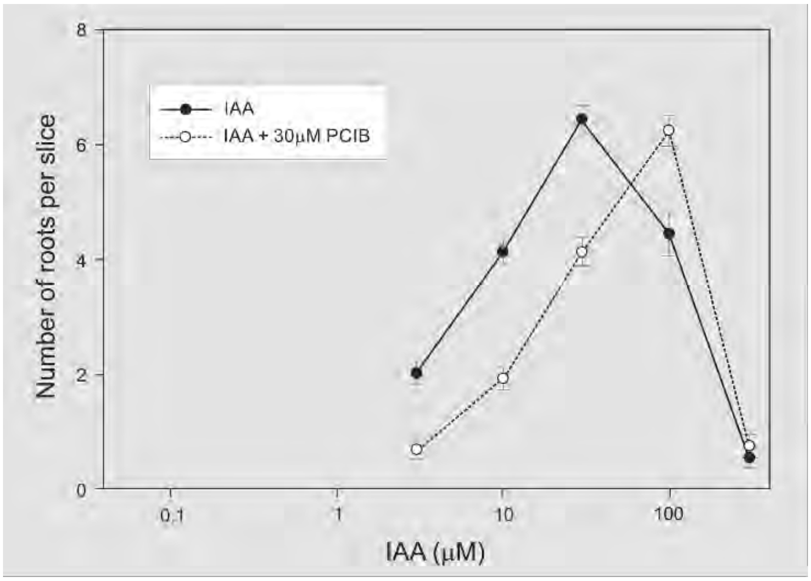
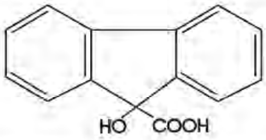


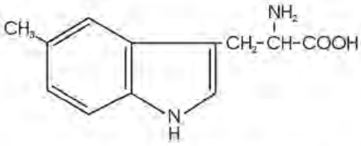
Fig. 5.5 The effect of IAA and PCIB on rooting of apple microcuttings (de Klerk *et al.,*unpublished).

whereas in culture of *Eleutherococcus senticosus,* TIBA suppressed embryo formation while cell division was not affected (Choi *et al.,* 2001).

Auxin polar transport was reported to be essential for bilateral symmetry during early plant embryogenesis. Three transport inhibitors (TIBA, *trans*-cinnamic acid (Table 5.2) and 9-hydroxyfluorene-9-carboxylic acid (16) induced formation of fused cotyledons in *in vitro* culture of early globular embryos of Indian mustard (Liu *et al.,* 1993). Also the symmetry of zygotic embryos is affected by auxin polar transport. Application of TIBA to wheat zygotic embryos cultivated *in vitro* caused abnormal symmetry, i.e. malformed embryos. The relative position of the shoot apical meristem was anomalous and no root meristem differentiated (Fischer and Neuhaus, 1996). In contrast, PCIB, which is considered to compete with auxin for the auxin receptor but to have no effect on auxin transport, did not affect embryo symmetry. ß-NAA, phenylpropionic acid, and 2-(*o*-chlorophenoxy)-2-methylpropionic acid, together with TIBA, prevented the re-callusing of indirectly formed *Sapindus trifoliatus* embryos, but did not permit embryo germination. Germination only occurred when 5-methyl-tryptophan (17) was added to the medium. The compound seemed to act as an anti-auxin (Desai *et al.,* 1986).



(16) 9-hydroxyfluorene-9-carboxylic acid



(17) 5-methyltryptophan

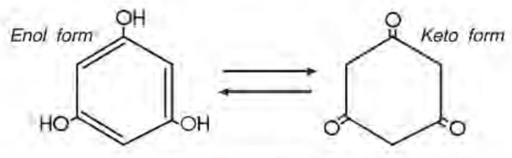
8. THE GROWTH REGULATORY EFFECTS OF PHENOLS

Compounds that carry one or more hydroxyl groups on an aromatic ring are termed phenolic compounds (for examples see Table 5.2). There are many papers on the growth regulating properties of phenolic compounds in tissue cultures. The documented effects of adding phenols to culture media are mainly an enhancement of callus growth, more effective adventitious shoot formation, the improved rooting of shoots, and a greater rate of shoot proliferation in certain shoot cultures. Most plant responses have involved a synergism with auxins, particularly IAA, so that a mode of action that is dependent on the regulation of internal IAA levels has seemed probable. Many mono-, di- and tri-hydroxyphenols and their more complex derivatives found naturally in plant cells are strong reducing agents and can serve as substrates for oxidative enzymes. This has led to two hypotheses as to their growth regulatory activity:

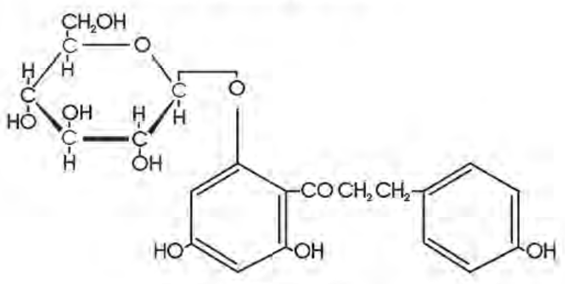
1. When added exogenously, hydroxyphenols act as alternative substrates for oxidative enzymes, and may protect auxin from oxidative breakdown (Stonier, 1971; Stonier *et al.,* 1970; James and Thurbon, 1981b. Lee and Skoog (1965) and Grambow and Langenbeck-Schwich (1983) reported that the substitution pattern of phenols affects the rate of IAA oxidation. Some monophenolics increase the rate, while some 3-substituted phenols depress it. Phenols were found to react with hydrogen peroxide produced during IAA degradation, thereby protecting the cell from its toxic effects. Relatively large amounts of natural inhibitors of IAA oxidase have been reported to be present in meristematic and juvenile tissues, but not in normal mature differentiated cells until they are wounded (Stonier and Yoneda, 1967; Stonier, 1969). The normal process of auxin (IAA) inactivation has also been reported to be inhibited in the callus produced following crown-gall infection, which is capable of autonomous growth in culture (Lipetz and Galston, 1959; Platt, 1954; Stonier, 1969; Bouillenne and Gaspar, 1970), and in auxin-habituated callus (Weis, 1967). Growing tobacco cells on media that favour the induction of auxin habituation (see Syono and Furuya, 1974) caused an increase in an inhibitor of auxin destruction (Syono, 1979). But some workers (*e*.g., Basu *et al.,* 1969; Hammerschlag, 1982) have questioned whether the stimulatory effect of phenols in promoting rooting is not due to some other function than that of preventing IAA destruction. Shoots of a non-rooting mutant of tobacco, *rac*, contain high levels of auxin protectors, namely chlorogenic acid and total soluble phenols (Faivre-Rampant *et al.,* 2000). Lee (1980) found that, in maize, some phenolic compounds can alter the relative proportions of free and bound IAA. On the other hand, enhancement of quercetin (Table 5.2) glucosylation by 2,4-D was described in *Vitis* cell cultures (Kokubo *et al.,* 2001).

2. Morphogenic activity is induced by the products formed when compounds such as phloroglucinol (18) and phloridzin (19) (see below) are oxidised. Gur *et al.*, (1988) advanced this hypothesis when they found that phloroglucinol only promoted rooting in apple clones with sufficient polyphenol oxidase activity to cause significant oxidation of the compound. Of significance to this proposal is the fact that phloroglucinol and phloridzin can inhibit vitrification, apparently by serving as precursors of lignin synthesis

It should be noted however that phenols may have other, unexpected roles. Thus, some phenolic compounds such as, or similar to, quercitin may act as true plant regulators – the phytotropins. Similarly, salicylic acid (Table 5.2) is now emerging as an important component of plant responses to biotic stress (see Chapter 7, Section 8).

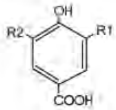
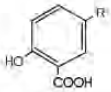
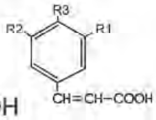
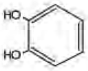
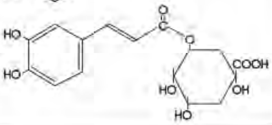
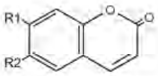
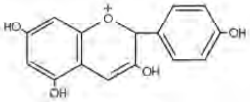
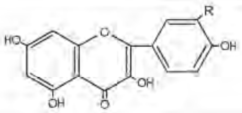
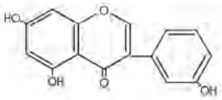
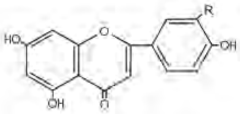


(18) phloroglucinol



(19) phloridzin

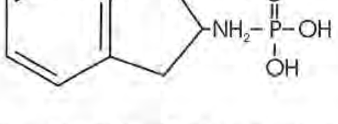
Table 5.2 Structures of Phenolic Compounds in Plants

Compound	Substitutions	Basic structure
Benzoic acids		
p-hydroxybenzoic acid	R1=R2=H	
protocatechuic acid	R1=H, R2=OH	
vanillic acid	R1=H, R2=OCH_3	
salicylic acid	R=H	
genistic acid	R=OH	
Cinnamic acids		
cinnamic acid	R1=R2=R3=H	
p-coumaric acid	R1=R2=H, R3=OH	
caffeic acid	R1=H, R2=R3=OH	
ferulic acid	R1=H, R2=OCH_3, R3=OH	
Dihydroxy phenols		
catechol		
chlorogenic acid		
Coumarins		
coumarin	R1=R2=H	
esculetin	R1=OH, R2=OH	
scopoletin	R1=OH, R2=OCH_3	
Anthocyanidins		
pelargonidin		
Flavonols		
kaempferol	R=H	
quercitin	R=OH	
Isoflavonols		
genistein		
Flavones		
apigenin	R=H	
luteolin	R=OH	

8.1. CORRELATIONS WITH ENDOGENOUS LEVELS

The natural rate of formation of some phenolic compounds has been observed to depend on the rate of growth of cultured tissues (Barz, 1977) and on auxin/cytokinin levels in the medium (Sargent and Skoog, 1960; Skoog and Montaldi, 1961). Their presence has frequently been correlated with the morphogenetic capacity of tissues (Tryon, 1956; Hackett, 1970; Kefeli and Kadyrov, 1971). A rise in the content of endogenous phenolics, including monoferuloyl, monocaffeoyl and mono-p-cumaroyl tartaric acids, was observed during root formation in *Vitis vinifera in vitro* (Mato et al., 1988). A correlation between flavonoid accumulation and root formation was also described in *Eucalyptus gunii* (Curir et al., 1990). Additionally, a correlation between level of endogenous derivatives of cinnamic and benzoic acids with embryogenesis was reported in *Medicago falcata* (Cvikrová et al., 1994).

Rawal and Mehta (1982) found that shoot formation from haploid tobacco callus occurred as the content of natural phenolic substances declined, while cellular differentiation occurred with increasing phenolic accumulation. Juvenile tissues naturally have high levels of auxin protectors (Stonier, 1971; 1972). Also, the level of aromatic amines, like tyramine or phenetylamine, seems to be correlated to morphogenetic events (Martin-Tanguy and Carre, 1993, Cvikrová *et al.,* 1996). On the other hand, a decreased content of phenolic acids (mainly derivatives of cinnamic acid (Table 5.2) found in alfalfa (*Medicago falcata* L.) cell suspension culture after treatment with an inhibitor of phenylalanine ammonia lyase (PAL), 2-aminoindan-2-phosphonic acid (AIP) (20), was connected with a decreased level of IAA, lower IAA-oxidase activity in later stages of the culture and with slower growth of the culture (Hrubcová *et al.,* 2000). In embryonic cultures of sessile oak, inhibiton of phenylpropanoid synthesis by AIP led to increased number of well developed somatic embryos (Cvikrová *et al.,* 2003).

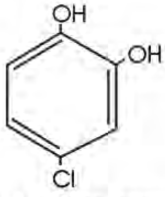


(20) aminoindan-2-phosphonic acid

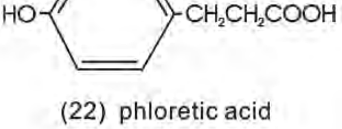
8.2. ROOT FORMATION

Some observations on natural levels of auxin protectors might suggest that their low levels are coupled with root initiation, but high levels with root growth. For example, shoots of apple grown *in vitro* were found to have low phenol contents at the root induction phase, but high contents as roots were growing (Druart *et al.,* 1982). In *Sequoiadendron giganteum*, phenolic compounds were found to decrease in concentration when shoots were moved to a root induction medium. The activity of peroxidases in the induction medium increased during 7-11 days and then decreased, roots appearing as phenols were decreasing (Monteuuis *et al.,* 1987). In each of these plants, peroxidase activity was inversely correlated with phenol content.

In chestnut, rhizogenesis occurred during an increase in the level of auxin protectors, whose basipetal transport was inhibited by applied IBA (Mato and Vieitez, 1986). The best time to explant shoot tips from adult chestnut material to a root-inducing medium, was during one of the first two peaks of growth of shoots, which coincided with the occurrence of maximum quantitites of natural phenolics (Chevre and Salesses, 1987). 4-chlororesorcinol (21), a polyphenol oxidase inhibitor (*i*.e.inhibiting the conversion of monophenols and di-hydric phenols to polyphenols) has been found to improve the rooting and subsequent growth of cuttings (Gad *et al.,* 1988). In shoot cuttings of *Cedrus deodara* adventitious rooting was stimulated both by IBA and by coumarin (Table 5.2) (Nandi *et al.,* 2002).



(21) 4-chlororesorcinol



(22) phloretic acid

8.3. EFFECT OF PHLOROGLUCINOL

Workers at East Malling Research Station discovered that in the genera *Malus* and *Prunus*, 1 mM phloroglucinol added to culture media containing growth substances, was able to enhance growth and the rate of axillary shoot production from shoot cultures (Jones, 1976; Jones, 1979). James and Wakerell (1982) found that the compound was without effect on the apple variety 'M26', but promotory on 'M9', while Whiteley and Abbott (1977) reported that the growth of shoot cultures of *Malus* 'Golden Delicious', 'Egremont Russet' and 'Bramley' was completely inhibited by 0.1-10 mM phloroglucinol. Hutchinson (1985) found that 1 mM phloroglucinol more than doubled the number of shoots produced by the apple cultivar 'Northern Spy' during the first two subcultures: there was no increase in shoot number by the fourth subculture.

Phloroglucinol and its analogues have proved to be effective in other plants of the Rosaceae. For example, added to the medium at 500 μM, phloroglucinol (and catechol – see below) increased adventitious shoot formation from *Rubus* callus (Compton and Preece, 1988). However stimulatory effects have also been reported on plants of some other families (*e*.g., on shoot growth and shoot proliferation of *Cinchona* (Rubiaceae) (Hunter, 1979; Krikorian *et al.,* 1982) and *Ficus carica* (Moraceae) (Pontikis and Melas, 1986). Vitrification is prevented by phloro-glucinol in an even wider range of plants. Adding phloridzin (19) and phloroglucinol to the medium, increased the number of somatic embryos produced from embryogenic callus of oil palm (Hanower and Hanower, 1984).

Rooting may be stimulated by phloroglucinol when added to rooting media together with auxin. This effect has been especially noted in several apple cultivars (Jones *et al.,* 1977, 1979; James and Thurbon, 1979, 1981 a,b; Jones and Hatfield, 1976; Zimmerman and Broome, 1981; Zimmerman, 1984). Welander and Huntrieser (1981) found that IBA plus 0.1 mM phoroglucinol promoted the rooting of adult shoots: 4.9 mM IBA plus 1 mM phloroglucinol induced the best rooting of juvenile shoots. Phloroglucinol and its analogues (see below) have been shown to promote rooting of *Prunus* (Chancel *et al.,* 1980), strawberry and *Rubus* genotypes in conjunction with IBA (but not NAA) (James, 1979; Sobczykiewicz, 1987). A promotive effect of phloroglucinol on rooting was also described in plantlet regeneration of *Ficus carica* from leaves (Yakushiji *et al.,* 2003), in micro-shoots of *Decalepis hamiltonii* (Reddy *et al.,* 2001) and in nodular callus of mangosteen (Te-chato and Lim, 1999). Interestingly, phloroglucinol is able to suppress accumulation of phenolic substances in the callus of water chestnut, enabling plant regeneration from the callus (Hoque and Arima, 2002).

The structurally-related glycoside, phloridzin (19), has the same effect as phloroglucinol (Jones, 1976) but it is heat labile and more expensive (Krikorian *et al.,* 1982). Phloridzin is metabolised into phloroglucinol and phloretic acid (22). Phloretic acid was also found by Jones and Hatfield (1976) to increase the proportion of apple shoots that could be rooted, but it was less active than phloroglucinol. Phloroglucinol had occasional inhibitory effects, *e*.g., on the rooting of sour cherry shoots grown *in vitro* (Snir, 1983) and in sentag shoots (Kooi *et al.,* 1999).

It has been suggested that phloroglucinol acts as a bactericide, increasing shoot regeneration only in shoot cultures carrying concealed bacterial infections. The stimulatory effect of the compound is, however, now thought to be largely independent of this effect (Jones and Hopgood, 1979; Jones and James, 1979). Most probably, the compound and its homologues act as auxin synergists (Hess, 1969), or auxin protectors (see above).

8.4. THE EFFECT OF CATECHOL

Catechol (Table 5.2) is another strong reducing agent that has been reported to regulate the rate of IAA oxidation in plant tissues. Hackett (1970) found that although apices of juvenile *Hedera* shoots would root in an irradiance of 53 - 70 $\mu mol.m^{-2}.s^{-1}$ when treated with 25-50 μM NAA, IAA had very little effect.

However, 60 μM IAA together with 50 μM catechol resulted in a rooting response that was almost equal to that produced by the optimum NAA level, suggesting that catechol protected IAA from light-induced degradation At low irradiance (7 $\mu mol.m^{-2}.s^{-1}$), IAA alone produced almost as many roots as IAA + catechol in high lighting. The rooting response of adult shoot tips in low light was very similar to that of juvenile tips in high light.

Catechol (and phloroglucinol, 500 μM), increased the number of adventitious shoots formed from leaf callus of tobacco (Compton and Preece, 1988). Catechol was much less effective than phloroglucinol in promoting the rooting of apple shoots (Jones and Hatfield, 1976) but promoted the rooting of etiolated *Populus robusta* cuttings in conjunction with IAA and sucrose (Pal and Nanda, 1981). This last test was not conducted under sterile conditions so that

catechol could have acted as an antiseptic. Poor rooting is a feature of contaminated cultures (Lang and Schwartz, 1981).

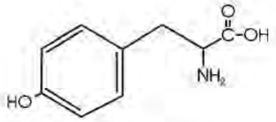
8.5. OTHER PHENOLIC COMPOUNDS

Like catechol, chlorogenic acid (Table 5.2) is a natural constituent of plants. It is a strong reducing agent, which has been noted to stimulate callus growth of *Prunus avium* stem segments (Feucht and Johal, 1977; Feucht and Schmid, 1980) and to promote the growth of olive callus (Lavee and Avidan, 1982). Together with 10 - 100 µM *p*-coumaric acid (Table 5.2), chlorogenic acid has been added routinely to media containing NAA and IBA to promote rooting of *Beta vulgaris* (Margara, 1977) and *Brassica* (Margara and Leydecker, 1978) shoots *in vitro*. Hammerschlag (1982) was able to root 100% of *Prunus cerasifera* shoots in the light when chlorogenic acid and IAA were present in the medium, but only 30% formed roots in response to IAA alone. Shoots kept in darkness all rooted in response to IAA alone.

Many natural coumarins are found in plants, but their biochemical or physiological roles are not well understood. Compounds of this kind have been found to affect a wide variety of processes, low levels sometimes exerting a stimulatory role, but higher levels are often inhibitory. This is particularly noticeable in the effect of coumarins on the activity of many classes of enzymes. Scopoletin (Table 5.2) has been reported to either increase or decrease IAA oxidase activity, according to concentration (Imbert and Wilson, 1970) and in tobacco callus it can inhibit IAA degradation (Skoog and Montaldi, 1961), perhaps by acting as a substrate for peroxidase enzymes.

Coumarin and related compounds, have been found to both stimulate and decrease protein synthesis, respiration and photophosphorylation and decrease carbohydrate metabolism (Brown, 1981), but increased growth in conjunction with IAA has been reported (Neumann, 1960). Adding 90-150 µM coumarin (and no other regulant) to Murashige and Tucker (1969) medium induced the formation of roots and shoots from stem sections of *Citrus* `Swingle Citrumelo' seedlings (Grosser and Chandler, 1986).

Tobacco pith callus grown on the medium of Linsmaier and Skoog (1965) with 12 µM IAA and 12 µM kinetin is wholly unorganised, but if grown on the same medium supplemented with 600 µM L-tyrosine (23), 80 µM adenine sulphate and 2.7 mM $NaH_2PO_4.H_2O$, adventitious shoots are formed (Thorpe and Murashige, 1968; 1970: Murashige, 1961). Tyrosine is a substrate for the enzyme phenylalanine ammonia lyase which converts it to p-coumaric acid. Perhaps the stimulation of adventitious shoot formation by tyrosine is therefore related to its conversion to phenolic acids that then protect, or interact with, auxin? Auxin requiring callus of tobacco has been found to accumulate more *p*-coumaric and *p*-ferulic acid (Table 5.2) than callus, which is auxin-habituated (*i*.e., auxin autotrophic) (Zador *et al.,* 1985).



(23) L- tyrosine

Other compounds, which have been suspected to be naturally-occurring inhibitors of IAA oxidase, may increase callus growth in certain circumstances. They include the quinone, juglone (Compton and Preece, 1988), some diphenolic flavonoids with antioxidant properties such as naringenin (Phillips, 1961, 1962), quercitin and its glycoside quercitrin (Furuya *et al.,* 1962; Thimann, 1963; Feucht and Nachit, 1978), catechin and flavandiols (Feucht and Nachit, 1977; Feucht and Schmid, 1980) and chemicals of the `β-inhibitor complex' (Bennett-Clark and Kefford, 1953). Examples of the latter are coumarin (see above), scopoletin (and its glucoside scopolin) (Schaeffer *et al.,* 1967), and various phenolic acids such as caffeic acid (Table 5.2), chlorogenic acid (see above), and sinapic acid (Thimann, 1963).

The phenolic vitamin riboflavin might be also of importance for plant development. It was shown to participate in the mechanism of colonization of alfalfa roots by *Sinorhizobium meliloti* (Yang *et al.,* 2002), to promote induction of embryogenic callus in *Zoysia japonica* (Asano *et al.,* 1996) and to protect auxin from oxidation (Brennan, 1996).

9. AUXIN –ETHYLENE INTERACTIONS

Higher auxin concentrations almost invariably increase ethylene production (see review by Kende, 1993). Ethylene accumulated in the tissue culture vessels may then inhibit the growth and development of many tissue culture grown plants (see Chapter 7). Conversely, ethylene may effect auxin transport and metabolism.

REFERENCES

ALONI R. 2004 The induction of vascular tissues by auxin. pp. 471-492 in Davies P.J. (ed.) *Plant Hormones*, Kluwer Academic Publishers, Dordrecht.

ASANO Y., KATSUMOTO H., INOKUMA C., KANEKO S., ITO Y. & FUJIIE A. 1996 Cytokinin and thiamine requirements and stimulative effects of riboflavin and alpha-ketoglutaric acid on embryogenic callus induction from the seeds of *Zoysia japonica* Steud. J. Plant Physiol. 149, 413-417.

BAKER D.A. 2000 Vascular transport of auxins and cytokinins in *Ricinus*. Plant Growth Regul. 32, 157-160.

BANDURSKI, R.S., COHEN, J.D., SLOVIN, J.P., REINECKE, D.M. 1995 Auxin biosynthesis and metabolism. pp. 39-65 in Davies P.J. (ed.) *Plant Hormones*. Kluwer Academic Publishers, Dordrecht.

BARENDSE G.W.M., CROES A.F., BOSVELD M., VAN DER KRIEKEN W.M.& WULLEMS, G.J. 1987 Uptake and metabolism of NAA and BAP in explants of tobacco in relation to *in vitro* flower bud formation. J. Plant Growth Regul. 6, 193-200.

BARNES L.R. 1979 *In vitro* propagation of water melon. Scientia Hort. 11, 223-227.

BARZ W. 1977 Catabolism of endogenous and exogenous compounds by plant cell cultures. pp. 153-171 in Barz *et al.* (eds.) 1977 (q.v.).

BARZ W., REINHARD E. & ZENK M.H. (eds.) 1977 *Plant Tissue Culture and its Biotechnological Application.* Springer-Verlag, Berlin, Heidelberg, New York.

BASU R.N., BOSE T.K., ROY B.N. & MUKHOPADHYAY A. 1969 Auxin synergists in rooting of cuttings. Physiol. Plant. 22, 649-652.

BAULCOMBE D.C. & KEY J.L. 1980 Polyadenylated RNA sequences which are reduced in concentration following auxin treatment of soybean hypocotyls. J. Biol. Chem. 255, 8907-8913.

BENNET-CLARK T.A. & KEFFORD N.P. 1953 Chromatography of the growth substances in plant extracts. Nature 171, 645-647.

BENNETT M.J., MARCHANT A., MAY S.T. & SWARUP R. 1998 Going the distance with auxin: unravelling the molecular basis of auxin transport. Phil. Trans. Roy. Soc. Lond. B, 353, 1511-1515.

BERTHON J.Y., BOYER N. & GASPAR T. 1991 Uptake, distribution and metabolism of 2,4-dichlorophenoxyacetic acid in shoots of juvenile and mature clones of *Sequoiadendron giganteum* in relation to rooting *in vitro*. Plant Physiol. Biochem. 29, 355-362.

BEYL C.A. & SHARMA G.C. 1983 Picloram induced somatic embryogenesis in *Gasteria* and *Haworthia*. Plant Cell Tiss. Organ Cult. 2, 123-132.

BHATLA S.C., KAPOOR S. & KHURANA J.P. 1996 Involvement of calcium in auxin-induced cell differentiation in the protonema of the wild strain and auxin mutants of the moss *Funaria hygrometrica*. J. Plant Physiol. 147, 547-552.

BLACKMON W.J., REYNOLDS B.D. & POSTEK C.E. 1981 Production of somatic embryos from callused cantaloupe hypocotyl explants. HortScience 16, 451 (Abst. 381).

BONGA J.M. & DURZAN D.J (eds.) 1982 *Tissue Culture in Forestry*. Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, Boston, Lancaster. ISBN 90-247-2660-3.

BOUILLENNE C. & GASPAR T. 1970 Auxin catabolism and inihibitors in normal and crown gall tissues of *Impatiens balsamina*. Can. J. Bot. 48, 1159-1163.

BOZHKOV P.V., FILONOVA L.H.& VON ARNOLD, S. 2002 A key developmental switch during Norway spruce somatic embryogenesis is induced by withdrawal of growth regulators and is associated with cell death and extracellular acidification. Biotechnol. Bioeng. 77, 658-667.

BRENNAN T.M. 1996 Decarboxylation of indole-3-acetic acid and inhibition of growth in *Avena sativa* seedlings by plant-derived photosensitizers. Photochem. Photobiol. 64, 1001-1006.

BRONSEMA F.B.F., REDIG P., VANOOSTVEEN W.J.F., VANONCKELEN H.A. & VAN LAMMEREN A.A.M. 1996 Uptake and biochemical analysis of 2,4-D in cultured zygotic embryos of *Zea mays* L. J. Plant Physiol. 149, 363-371.

BROWN S.A. 1981 Coumarins. pp. 269-300 in Conn E.E. (ed.) 1981 (q.v.).

BUCHANAN B.B., GRUISSEN W., JONES R.L. 2000. *Biochemistry and Molecular Biology of Plants*. American Society of Plant Physiologists, Rockville, Maryland.

CAMPBELL A.D. & SUTTER E.G. 1986 Changes in IAA concentration in agar during tobacco callus culture. p. 297 in Somers *et al*. (eds.) 1986 (q.v.).

CARMAN J.G., JEFFERSON N.E. & CAMPBELL W.F. 1988 Induction of embryogenic *Triticum aestivum* L. calli. II. Quantification of organic addenda and other culture variable effects. Plant Cell Organ Tiss. Cult. 12, 97-110.

CASSELLS A.C. 1979 The effect of 2,3,5-tri-iodobenzoic acid on caulogenesis in callus cultures of tomato and *Pelargonium*. Physiol. Plant. 46, 159-164.

CASSELLS A.C., LONG R.D. & MOUSDALE D.M.A. 1982 Endogenous IAA and morphogenesis in tobacco petiole cultures. Physiol. Plant. 56, 507-512.

CENTENO M.L., RODRIGUEZ A., FEITO I. & FERNANDEZ B. 1996 Relationship between endogenous auxin and cytokinin levels and morphogenic responses in *Actinidia deliciosa* tissue cultures. Plant Cell Rep. 16, 58-62.

CHANCEL L.M., MACHIEX J. & JONARD R. 1980 Les conditions du microbouturage *in vitro* du pecher (*Prunus persica* Bathsch.): influences combine des substances de croissance et de divers composs phenoliques. Physiol. Veg. 18, 597-608.

CHANG F.Y. & VANDEN BORN W.H. 1971 Dicamba uptake, translocation, metabolism and selectivity. Weed Sci. 19, 113-117.

CHATURVEDI H.C., SHARMA A.K. & PRASAD R.N. 1978 Shoot apex culture of *Bougainvillea glabra* `Magnifica'. HortScience 13, 36.

CHENG T.-Y. 1972 Induction of IAA synthesis in tobacco pith explants. Plant Physiol. 50, 723-727.

CHEVRE A.-M. & SALESSES G. 1987 Choice of explants for chestnut micropropagation. Acta Hort. 212, 517-523.

CHOI Y.E., KATSUMI M.& SANO, H. 2001 Triiodobenzoic acid, an auxin polar transport inhibitor, suppresses somatic embryo formation and postembryonic shooot/root development in *Elutherococcus senticosus*. Plant Sci. 160, 1183-1190.

CLAPHAM D. 1973 Haploid *Hordeum* plants from anthers *in vitro*. Z. Pflanzenzucht. 69, 142-155.

CLARE M.V. & COLLIN H.A. 1974 The production of plantlets from tissue cultures of Brussels sprout (*Brassica oleracea* L. var. *gemmifera* D.C.). Ann. Bot. 38, 1067-1076.

CLELAND R.E. 2004 Auxin and cell elongation. pp. 204-220 in Davies P.J. (ed.) *Plant Hormones*, Kluwer Academic Publishers, Dordrecht.

CLINE M.G. 1994 The role of hormones in apical dominance - new approaches to an old problem in plant development. Physiol. Plant.90, 230-237.

COHEN J.D. & BANDURSKI R.S. 1982 Chemistry and physiology of the bound auxins. Ann. Rev. Plant Physiol. 33, 403-430.

COMPTON M.E. & PREECE J.E. 1988 Effects of phenolic compounds on tobacco callus and blackberry shoot cultures. J. Am. Soc. Hort. Sci. 113, 160-163.

CONN E.E. (ed.) 1981 *The Biochemistry of Plants*. Vol. 7. *Secondary Plant Products*. Academic Press, New York, London, Toronto. ISBN 0-12-675407-1

COONEY T.P. & NONHEBEL, H.M. 1991. Biosynthesis of indole-3-acetic acid in tomato shoots – measurement, mass spectral identification and incorporation of H-2 from H_20-H-2 into indole-3-acetic acid, D-tryptophan and L-tryptophan, indole-3-pyruvate and tryptamine. Planta 184, 368-376

COX D.N. & MUDAY G.K. 1994 NPA binding activity is peripheral to the plasma membrane and is associated with the cytoskeleton. Plant Cell 6, 1941-1953.

CURIR P., VANSUMERE C.F., TERMINI A., BARTHE P., MARCHESINI A. & DOLCI M. 1990 Flavonoid accumulation is correlated with adventitious root formation in *Eucalyptus gunnii* Hook micropropagated through axillary bud stimulation. Plant Physiol. 92, 1148-1153.

CVIKROVÁ M., MALÁ J., HRUBCOVÁ M., EDER J., ZON J.& MACHÁČKOVÁ, I. 2003 Effect of inhibition of biosynthesis of phenylpropanoids on sessile oak somatic embryogenesis.- Plant Physiol. Biochem. 41, 251-259.

CVIKROVA M., HRUBCOVA. M, EDER J. & BINAROVA P. 1996 Changes in the levels of endogenous phenolics, aromatic monoamines, phenylalanine ammonia-lyase, peroxidase and auxin oxidase activities during initiation of alfalfa embryogenic and non-embryogenic calli. Plant Physiol. and Biochem. 34, 853-861.

DAVIES, P.J. (ed.) 1995. *Plant Hormones. Physiology, Biochemistry and Molecular Biology*, 2nd.Ed., Kluwer Acad. Publ., Dordrecht, The Nethrelands.

DAVIES P.J. 2004 Regulatory factors in hormone action: Level, location and signal transduction. pp. 16-35 in: Davies P.J. (ed.) *Plant Hormones*, Kluwer Academic Publishers, Dordrecht..

DAVEY M.R., FOWLER M.W. & STREET H.E. 1971 Cell clones contrasted in growth, morphology and pigmentation isolated from a callus culture of *Atropa belladonna* var. *lutea*. Phytochem. 10, 2559-2575.

DELBARRE A., MULLER P., IMHOFF V. & GUERN J. 1996 Comparison of the mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxyacetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells. Planta 198, 532-541.

DELBARRE A., MULLER P., PARRY G., SWARUP R. MARCHANT A., BENNETT M. & PERROT-RECHENMANN C. 2000 Characterisation and physiological effects of auxin influx carrier inhibitors. pp. 14-15 in *EMBO Workshop on Auxin,* 13-19 May 2000, Calcatoggio, France.

DESAI H.V., BHATT P.N. & MEHTA A.R. 1986 Plant regeneration of *Sapindus trifoliatus* L. (soapnut) through somatic embryogenesis. Plant Cell Rep. 5, 190-191.

DHARMASIRI N & ESTELLE M. 2004. Auxin signaling and regulated protein degradation. Trends Plant Sci. 9: 302-308.

DOBREV P., PETKOV P. SVETLEVA D., IVANOVA A., DJILIANOV D., PETKOVA S. & ATANASSOV A. 2001 Analysis of some endogenous plant hormones during induction of somatic embryogenesis in common bean (*Phaseolus vulgaris* L.). Biotechnol. Biotechnol. Equip. 15, 17-22.

DRUART Ph., KEVERS Cl., BOXUS Ph. & GASPAR Th. 1982 *In vitro* promotion of root formation by apple shoots through darkness effect on endogenous phenols and peroxidases. Z. Pflanzenphysiol. 108, 429-436.

DUNLAP J.R., KRESOVICH S. & McGEE R.E. 1986 The effect of salt concentration on auxin stability in culture media. Plant Physiol. 81, 934-936.

ECKARDT N.A. 2001 New insights into auxin biosynthesis. Plant Cell 13, 1-3.

EDWARDS K.L. & GOLDSMITH M.H.M. 1980 pH-dependent accumulation of indoleacetic acid by corn coleoptile sections. Planta 147, 457-466.

ELLIOT M.C., MALONEY M.M. & HALL J.F. 1978 Auxin relations in Sycamore cell suspension cultures. Plant Physiol. 61, (Suppl.) 45 (Abst. 244).

ENGVILD K.C. 1985 Pollen irradiation and possible gene transfer in *Nicotiana* species. Theor. Appl. Genet. 69, 457-461.

ESTELLE M. 1999. Auxin perception and signal transduction. - pp. 411-420 in Hooykaas P.J.J., Hall M.A., Libbenga K.R. (eds.) *Biochemistry and Molecular Biology of Plant Hormones*. Elsevier, Amsterdam.

FAIVRE-RAMPANT O., KEVERS C. & GASPAR, T. 2000 IAA-oxidase activity and auxin protectors in nonrooting, rac mutant shoots of tobacco *in vitro*. Plant Sci. 153, 73-80.

FAKHRAI H., FAKHRAI F. & EVANS P.K. 1989 *In vitro* culture and plant regeneration in *Vicia faba* subsp. *equina* (var. Spring Blaze). J. Exp. Bot. 40, 813-817.

FEHER A., PASTERNAK T., OTVOS K., MISKOLCZI P. & DUDITS D. 2002 Induction of embryogenic competence in somatic plant cells: a review. Biologia 57, 5-12.

FENG K. & LINCK A.J. 1970 Effects of N-1-naphthylphthalamic acid on the growth and bud formation of tobacco callus grown *in vitro*. Plant Cell Physiol. 11, 589-598.

FEUCHT W. & JOHAL C.S. 1977 Effect of chlorogenic acids on the growth of excised young stem segments of *Prunus avium*. Acta Hort. 78, 109-114.

FEUCHT W. & NACHIT M. 1977 Flavolans and growth-promoting catechins in young shoot tips of *Prunus* species and hybrids. Physiol. Plant. 40, 230-234.

FEUCHT W. & NACHIT M. 1978 Flavenol glycosides of different species and hybrids from the *Prunus* section *Eucerasus* and the growth-promoting activity of quercetin derivatives. Scientia Hort. 8, 51-56.

FEUCHT W. & SCHMID P.P.S. 1980 Effect of ortho-dihydroxy-phenols on growth and protein pattern of callus cultures. Physiol. Plant. 50, 309-313.

FIOLA J.A., CHIN C. & POLLACK B.L. 1978 Effect of a-(p-chlorophenoxy) isobutyric acid on regeneration of eggplant from callus tissue. HortScience 13, 354 (Abst. 125).

FIND J., GRACE L. & KROGSTRUP P. 2002 Effect of anti-auxins on maturation of embryogenic tissue cultures of Nordmanns fir (*Abies nordmanniana*). Physiol. Plant. 116, 231-237.

FISCHER C. & NEUHAUS G. 1996 Influence of auxin on the establishment of bilateral symmetry in monocots Plant J. 9, 659-669.

FOREST J.C. & WIGHTMAN F. 1972. Amino acid metabolism in plants. III. Purification and some properties of a multispecific aminotransferase isolated from bushbean seedlings (*Phaseolus vulgaris* L.). Can. J. Biochem. 50, 813-829.

FRIEDMAN R., ALTMAN A. & BACHRACH U. 1985 Polyamines and root formation in mung bean hypocotyl cuttings. II. Incorporation of precursors into polyamines. Plant Physiol. 79, 80-83.

FRIML J. & PALME K. 2002. Polar auxin transport - old questions and new concepts? Plant Mol. Biol. 49, 273-284.

FRIML, J. 2003. Auxin transport – shaping the plant. Curr. Opin. Plant. Biol. 6, 1-6.

FUJIMURA T. & KOMAMINE A. 1979 Involvement of endogenous auxin in somatic embryogenesis in a carrot cell suspension culture. Z Pflanzenphysiol. 95, 13-19.

FUJISAWA Y., KATO H., IWASAKI Y. 2001. Structure and function of heterotrimeric G proteins in plants. Plant Cell Physiol. 42, 789-794.

FUJIWARA A. (ed.) 1982 *Plant Tissue Culture* 1982. Proc. 5th. Int. Cong. Plant Tiss. Cell Cult., Japan. Assoc. Plant Tissue Culture, Japan.

FUNADA R., KUBO T., TABUCHI M., SUGIYAMA T. & FUSHITANI M. 2001 Seasonal variations in endogenous indole-3-acetic acid and abscisic acid in the cambial region of *Pinus densiflora* Sieb. et Zucc. stems in relation to early-wood/latewood transition and cessation of tracheid production. Holzforschung 55, 128-134.

FURUYA M., GALSTON A.W. & STOWE B.B. 1962 Isolation from peas of co-factors and inhibitors of indole-3-acetic acid oxidase. Nature 193, 456-457.

GAD A.E., BEN-EFRAIM I., YAVZURY M., WEINBERG C. & FRIEDMAN G. 1988 Improvement of cutting quality by 4-chlororesorcinol. Comb. Proc. Int. Plant Prop. Soc. 37, 119-123.

GALWEILER L., CHANGHUI G., MULLER A., WISMAN E., MENDGEN K., YEPHREMOV A. & PALME K. 1998 Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. Science 282, 2226-2230.

GENOVESI A.D. & COLLINS G.B. 1982 *In vitro* production of haploid plants of corn via anther culture. Crop Sci. 22, 1137-1144.

GIBSON R.A., SCHNEIDER E.A. & WIGHTMAN F. 1972 Biosynthesis and metabolism of indolyl-3-acetic acid. II. *In vivo* experiments with 14C-labelled precursors of IAA in tomato and barley shoots. J. Exp. Bot. 23, 381-399.

GOLDSMITH M.H.M. 1977 The polar transport of auxin. Annu. Rev. Plant Physiol. 28, 439-478.

GRAMBOW H.J. & LANGENBECK-SCHWICH B. 1983 The relationship between oxidase activity, peroxidase activity, hydrogen peroxide, and phenolic compounds in the degradation of indole-3-acetic acid *in vitro*. Planta 157, 131-137.

GRAY D.J. & CONGER B.V. 1985 Influence of dicamba and casein hydrolysate on somatic embryo number and culture quality in cell suspensions of *Dactylis glomerata* (Gramineae). Plant Cell Tiss. Organ Cult. 4, 123-133.

GROSSER J.W. & CHANDLER J.L. 1986 *In vitro* multiplication of Swingle citrumelo rootstock with coumarin. HortScience 21, 518-520.

GRUNEWALDT J. 1977 Adventivknospenbildung und Pflanzenregeneration bei Gesneriaceae *in vitro*. Gartenbauwiss. 42, 171-175.

GUILFOYLE T.J, & HAGEN G. 2001. Auxin response factors. J. Plant Growth Regul. 20: 281-291.

GUILFOYLE T.J. 1999 Auxin-regulated genes and promoters. pp. 423-459.in Hooykaas P.J.J., Hall M.R. & Libbenga K.R. (eds) *Biochemistry and Molecular Biology of Plant Hormones*. Elsevier Science B.V., Amsterdam.

GUAN H.Y. & DE KLERK G-J. 2000 Stem segments of apple microcuttings take up auxin predominantly via the cut surface and not via epidermal surface. Sci. Hort. 86, 23-32.

GUAN H.Y., HUISMAN P. & DE KLERK, G.-J. 1997 Rooting of apple stem slices *in vitro* is affested by indoleacetic-acid depletion of the medium. Angewandte Botanik 71, 80-84.

GUR A., GAD A.E. & HAAS E. 1988 Rooting of apple rootstock clones as related to phenols and their oxidation. Acta Hort. 227, 160-166.

HACKETT W.P. 1970 The influence of auxin, catechol and methanolic tissue extracts on root initiation in aseptically cultured shoot apices of the juvenile and adult forms of *Hedera helix*. J. Am. Soc. Hort. Sci. 95, 398-402.

HAGEN G., GUILFOYLE T.J. & GRAY W.M. 2004 Auxin signal transduction. pp. 282-303 in Davies P.J. (ed.) *Plant Hormones*. Kluwer T. Academic Publishers, Dordrecht.

HAGER A., MENZEL H. & KRAUSS A. 1971 Versuche und Hypothese zur Primärwirkung des Auxins bei Streckungswachstum. Planta 100, 47-75.

HAMMERSCHLAG F. 1982 Factors influencing *in vitro* multiplication and rooting of the plum rootstock myrobalan (*Prunus cerasifera* Ehrh.). J. Am. Soc. Hort. Sci. 107, 44-47.

HANOWER J. & HANOWER P. 1984 Inhibition et stimulation en culture *in vitro* de l'embryogenèse des souches issues d'explant foliaires de palmier à l'huile. Compt. Rend. Acad. Sci. Paris 298, ser. III 45-48.

HARAMAKI C. 1971 Tissue culture of *Gloxinia* Comb. Proc. Int. Plant Prop. Soc. 21, 442-448.

HELLMANN H. & ESTELLE M. 2002 Plant development: Regulation by protein degradation. Science 297, 793-797.

HEMERLY A., FERREIRA P., ENGLER J., ENGLER G., INZE D. & VANMONTAGU M. 1993 The control of cell cycle in *Arabidopsis* plant cell cultures. J. Plant Res. 51-56 Sp. Iss. 3.

HENKE R.R., HUGHES, K.W., CONSTANTIN M.J., HOLLAENDER A.A. & WILSON C.M. (eds.) 1985 *Tissue Culture in Forestry and Agriculture*. Plenum Press, New York, London.

HESS C.E. 1969 Internal and external factors regulating root initiation. pp. 42-53 in Whittingham W.J. (ed.) 1969 *Root Growth*. Butterworths, London.

HEYSER J.W., DYKES T.A., DEMOTT K.J. & NABORS M.W. 1983 High frequency, long-term regeneration of rice from callus culture. Plant Sci. Lett. 29, 175-182.

HILDEBRANDT A.C., WILMAR J.C., JOHNS H. & RIKER A.J. 1963 Growth of edible chlorophyllous plant tissues *in vitro*. Am. J. Bot. 50, 248-254.

HINMAN R.L. & LANG J. 1965 Peroxidase-catalyzed oxidation of indole-3-acetic acid. Biochemistry 4, 144-158.

HOPKINS W.G. 1995 *Introduction to Plant Physiology*. John Wiley & sons, New York, pp. 464.

HOQUE A. & ARIMA S. 2002 Overcoming phenolic accumulation during callus induction and *in vitro* organogenesis in water chestnut (*Trapa japonica* Flerov). In Vitro Cell Develop. Biol. 38, 342-346.

HRUBCOVA M., CVIKROVA M., EDER J., ZON J. & MACHACKOVA I. 2000 Effect of inhibition of phenylpropanoid biosynthesis on peroxidase and IAA-oxidase activities

and auxin content in alfalfa suspension cultures. Plant Physiol. Biochem. 38, 949-956.

HUNTER C.S. 1979 *In vitro* culture of *Cinchona ledgeriana* L. J. Hort. Sci. 54, 111-114.

HUTCHINSON J.F. 1985 Effect of explant type on shoot proliferation and physical support on root initiation for a range of horticultural species. pp. 327-328 in Henke *et al*. (eds.) 1985 (q.v.).

IMBERT M.P. & WILSON L.A. 1970 Stimulatory and inhibitory effects of scopoletin on IAA oxidase preparations from sweet potato. Phytochem. 9, 1787-1794.

IMHOFF V., MULLER P., GUERN J. & DELBARRE A. 2000 Inhibitors of the carrier-mediated influx of auxin in suspension-cultured tobacco cells. Planta 210: 580-588.

IRVINE J.E., FITCH M. & MOORE P.H. 1983 The induction of callus in sugarcane tissue cultures by selected chemicals. Plant Cell Tiss. Organ Cult. 2, 141-149.

ISHIKAWA, K., KAMADA, H., YAMAGUCHI, I., TAKAHASHIO, N. & HARADA, H. 1988. Morphology and hormone levels of tobacco and carrot tissues transformed by *Agrobacterium tumefaciens*. 1. Auxin and cytokinin contents of cultured tissue transformed with wild-type and mutant Ti plasmid. Plant Cell Physiol. 29: 461-466

JAMES D.J. & THURBON I.J. 1979 Rapid *in vitro* rooting of the apple rootstock M.9. J. Hort. Sci., 54, 309-311.

JAMES D.J. & THURBON I.J. 1981a Phenolic compounds and other factors controlling rhizogenesis *in vitro* in the apple rootstocks M.9 and M.26. Z. Pflanzenphysiol. 105, 11-20.

JAMES D.J. & THURBON I.J. 1981b Phenolic compounds and other factors controlling rhizogenesis *in vitro* in the apple rootstocks M.9 and M.26. Z. Pflanzenphysiol. 105, 11-20.

JAMES D.J. & WAKERELL I.J. 1982 The control of rhizogenesis *in vitro* in difficult-to-root apple rootstocks. pp. 187-188 in Fujiwara A. (ed.) 1982 (q.v.).

JAMES D.J. 1979 The role of auxins and phloroglucinol in adventitious root formation in *Rubus* and *Fragaria* grown *in vitro*. J. Hort. Sci. 54, 273-277.

JAMES D.J. & THURBON I.J. 1981a Phenolic compounds and other factors controlling rhizogenesis *in vitro* in the apple rootstocks M.9 and M.26. Z. Pflanzenphysiol. 105, 11-20.

JAMES D.J. & THURBON I.J. 1981b Phenolic compounds and other factors controlling rhizogenesis *in vitro* in the apple rootstocks M.9 and M.26. Z. Pflanzenphysiol. 105, 11-20.

JARRET R.L., FISHER J.B. & LITZ R.E. 1985 Organ formation in *Musa* tissue cultures. J. Plant Physiol. 121, 123-130.

JELASKA S., RENGEL Z. & CESAR V. 1984 Plant regeneration from mesocotyl callus of *Hordeum vulgare* L. Plant Cell Rep. 3, 125-129.

JOHN P.C.L., ZHANG K., DONG C., DIEDERICH L. & WIGHTMAN F. 1993 P34(CDC2) related proteins in control of cell-cycle progression. The switch between division and differentiation in tissue development and stimulation and division by auxin and cytokinin. Austr. J. Plant Physiol. 20, 503-526.

JONES A.M. 1994 Auxin-binding proteins. Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 393-420.

JONES O.P. & HATFIELD S.G.S. 1976 Root initiation in apple shoots cultured *in vitro* with auxins and phenolic compounds. J. Hort. Sci. 51, 495-499.

JONES O.P. & HOPGOOD M.E. 1979 The successful propagation *in vitro* of two rootstocks of *Prunus*: the plum rootstock Pixy (*P. institia*) and the cherry rootstock F.12/1 (*P. avium*). J. Hort. Sci. 54, 63-66.

JONES O.P. & JAMES D.J. 1979 Propagation *in vitro* of apple and other woody fruit plants. In Vitro 15, 210 (Abst.195).

JONES O.P. 1976 Effect of phloridzin and phloroglucinol on apple shoots. Nature 262, 392-393; 724.

JONES O.P. 1979 Propagation *in vitro* of apple trees and other woody fruit plants: methods and applications. Sci. Hort. 30, 44-48.

JONES O.P., HOPGOOD M.E. & O'FARRELL D. 1977 Propa-gation *in vitro* of M.26 apple root stocks. J. Hort. Sci. 52, 235-238.

JONES O.P., PONTIKIS C.A. & HOPGOOD M.E. 1979 Propagation *in vitro* of five apple scion cultivars. J. Hort. Sci. 54, 155-158.

JOUANNEAU J.P. 1971 Côntrole par les cytokinines de la synchronisation des mitoses dans les cellules de tabac. Exp. Cell Res. 67, 329-337.

KAPARAKIS G. & ALDERSON P.G. 2003 Differential callus type formation by auxins and cytokinin in *in vitro* cultures of pepper (*Capsicum annuum* L.).- Plant Biosystems 137, 275-280.

KEFELI V.I. & KADYROV C.Sh. 1971 Natural growth inhibitors, their chemical and physiological properties. Annu. Rev. Plant Physiol. 22, 185-196.

KEFFORD N.P. & CASO O.H. 1972 Organ regeneration on excised roots of *Chondrilla juncea* and its chemical regulation. Aust. J. Biol. Sci. 25, 691-706.

KENDE H. 1993 Ethylene biosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 283-307.

KEPINSKI, S. & LEYSER, O. 2002. Ubiquitination and auxin signaling: a degrading story. Plant Cell 14: 81-95.

KEVERS C., GASPAR T., DOMMES J. 2002 The beneficial role of different auxins and polyamines at successive stages of somatic embryo formation and development of *Panax ginseng in vitro*. Plant Cell Tiss. Org. Cult. 70, 181-188.

KIM Y.S., HAHN E.J., YEUNG E.C. & PAEK, K.Y. 2003 Lateral root development and saponin accumulation as affected by IBA or NAA in adventitious root cultures of *Panax ginseng* CA Meyer. In Vitro Cell. Develop. Biol. 39, 245-249.

KIYOSUE T., KAMADA H. & HARADA H. 1989 Induction of somatic embryogenesis from carrot seed by hypochlorite treatment. Plant Tiss. Cult. Lett. 6, 138-143.

KIYOSUE T., TAKANO K., KAMADA H. & HARADA H. 1990 Induction of somatic embryogenesis in carrot by heavy metal ions. Can. J. Bot. 68, 2301-2303.

KLEE H.J., HORSCH R.B., HINCHEE M.A., HEIN M.B. & HOFFMANN N.L. 1987 The effects of overproduction of 2 *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic *Petunia* plants. Genes & Develop. 1, 86-96.

KLEMS M., TRUKSA M., MACHACKOVA I., EDER J. & PROCHAZKA S. 1998 Uptake, transport and metabolism of C^{14}-2,4-dichlorophenoxyacetic acid (C^{14}-2,4-D) in cucumber (*Cucumis sativus* L.) explants Plant Growth Regul. 26, 195-202.

KOENS K.B., NICOLOSO F.T., VAN VLIET T.B., HARTEVELD M., BOOT C.J.M., VAN IREN F., MULDER P., LIBBENGA K.R. & KIJNE J.W. 1995 Kinetics of 2,4-dichlorophenoxyacetic acid content in an auxin-dependent suspension culture of *Nicotiana tabacum* cells J. Plant Physiol. 147, 383-390.

KOKUBO T., AMBE-ONO Y., NAKAMURA M., ISHIDA H., YAMAKAWA T. & KODAMA T. 2001 Promotive effect of auxins on UDP-glucose: Flavonol glucosyltransferase activity in *Vitis* sp cell cultures. J. Biosci. Bioeng. 91, 564-569.

KOOI L.T., KENG C.L. & HOE C.T.K. 1999 *In vitro* rooting sentang shoots (*Azadirachta excelsa* L.) and acclimatization of the plantlets. In Vitro Cell. Develop. Biol. 35, 396-400.

KOVTUN Y., CHIU W.-L., ZENG W. & SHEEN J. 1998 Suppression of auxin signal transduction by a MAPK cascade in higher plants. Nature 395, 716-720.

KRIKORIAN A.D., SINGH M. & QUINN C.E. 1982 Aseptic micropropagation of *Cinchona*: Prospects and problems. pp. 167-174 in Rao A.N. (ed.) 1982 *Tissue Culture of Economically*

Important Plants. Proc. Int. Symp., Singapore, 1981. COSTED and ANBS Publications.

KUTÁČEK M. 1985 Auxin biosynthesis and its regulation on the molecular level. Biol. Plant. 27, 145-153.

LANG G.A. & SCHWARTZ R.F. 1981 Effects of a bacterial contamination on rooting and growth of *in vitro Nephrolepsis* cultures, and isolation and identification of causal organism. HortScience 16, 463 (Abst. 471).

LAVEE S. & AVIDAN N. 1982 Growth responses of tree callus to chlorogenic acid and related phenolic substances. pp. 165-168 in Fujiwara A. (ed.) 1982 (q.v.).

LAW D.M. & DAVIES P.J. 1990 Comparative indole-3-acetic acid levels in the slender pea and other pea phenotypes. Plant Physiol. 93, 1539-1543.

LAZZERI P.A., HILDEBRAND D.F., SUNEGA J., WILLIAMS E.G. & COLLINS G.B. 1988 Soybean somatic embryogenesis: interactions between sucrose and auxin. Plant Cell Rep. 7, 517-520.

LEE T.T. & SKOOG F. 1965 Effects of hydroxybenzoic acids on indoleacetic acid inactivation by tobacco callus extracts. Physiol. Plant. 18, 577-585.

LEE T.T. 1980 Effects of phenolic substances on metabolism of exogenous indole-3-acetic acid in maize stems. Physiol. Plant. 50, 107-112.

LELJAK-LEVANIC D., BAUER N., MIHALJEVIC S. & JELASKA, S. 2004 Changes in DNA methylation during som-atic embryogenesis in *Cucurbita pepo* L. Plant Cell Rep. 23, 120-127.

LEYSER H.M.O., PICKETT F.B., DHARMASIRI S. & ESTELLE M. 1996 Mutation in AXR3 gene of *Arabidopsis* result in altered auxin response including ectopic expression from the SAUR-AC1 promoter. Plant J. 10, 403-413.

LEYSER O. 2001. Auxin signalling: the beginning, the middle and the end. Curr. Op. Plant Biol. 4, 382-386.

LI Y., SHI, X.Y., STRABALA, T.J., HAGEN, G. & GUILFOYLE, T.J. 1994. Transgenic tobacco plants that over-produce cytokinins show increased tolerance to exogenous auxin and auxin transport inhibitors. Plant Science 100, 9-14

LIBBENGA K.R. & MENNES A.M. 1995 Hormone binding and signal transduction. pp. 272-297 in Davies P.J. (ed.) *Plant Hormones: Physiology, Biochemistry and Molecular Biology.* 2nd edn., Kluwer Academic Publishers, Dordrecht, Boston, London.

LINSMAIER E.M. & SKOOG F. 1965 Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18, 100-127.

LIPETZ J. & GALSTON A.W. 1959 Indole acetic acid oxidase and peroxidase activities in normal and crown gall tissue cultures of *Parthenocissus tricuspidata*. Am. J. Bot. 46, 193-196.

LIU C.M., XU Z.H. & CHUA N.H. 1993 Polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. Plant Cell 5, 621-630,

LJUNG K, HULL AK, KOWALCZYK M, MARCHANT A, CELENZA J, COHEN JD, SANDBERG G. 2002. Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana.* Plant Mol. Biol. 49, 249-272.

LO SCHIAVO F., PITTO L., GIULIANO G., TORTI G., NUTI-RONCHI V., MARAZZITI D., VERGARA R., ORSELLI S. & TERZI M. 1989 DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. Theor. Appl. Genet. 77, 325-331.

LOMAX T.L., MUDAY G.K & RUBERY P.H. 1995 Auxin transport. pp. 509-530 in Davies P.J. (ed.) *Plant Hormones: Physiology, Biochemistry and Molecular Biology*. 2nd edn., Kluwer Academic Publishers, Dordrecht, Boston, London.

LUDWIG-MULLER J. & EPSTEIN E. 1991 Occurrence and *in vivo* biosynthesis of indole 3-butyric acid in corn (*Zea mays* L.). Plant Physiol. 97: 765-770.

MAEDA E. & THORPE T.A. 1979 Effects of various auxins on growth and shoot formation in tobacco callus. Phytomorph. 29, 146-155.

MAGNUS V. NIGOVIC B., HANGARTER R.P. & GOOD N.E. 1992 N-(indol-3-ylacetyl)amino acids as sources of auxin in plant tissue culture. J. Plant Growth Regul. 11, 19-28

MARGARA J. & LEYDECKER M.-T. 1978 Differents types d'organogense observe chez le Colza, *Brassica napus* L. var. *oleifera* Metzg. Compt. Rend. Acad. Sci. Paris 287D, 17-20.

MARGARA J. 1977 Effets d'auxines et d'antiauxines sur la néoformation de bourgeons *in vitro* chez le chou-fleur (*Brassica oleracea* L. var. *botrytis*). Compt. Rend. Acad. Sci. Paris 284D, 1883-1885.

MARTIN-TANGUY J. & CARRE M. 1993 Polyamines in grape-vine microcuttings cultivated *in vitro*. Effects of amines and inhibitors of polyamine biosynthesis on polyamine levels and microcutting growth and development. Plant Growth Regul. 13, 269-280.

MATO M.C., RUA M.L. & FERRO, E. 1988 Changes in levels of peroxidases and phenolics during root formation in *Vitis* cultured *in vitro*. Physiol. Plant. 72, 84-88.

MATO M.C. & VIEITEZ A.M. 1986 Changes in auxin protectors and IAA oxidase during the rooting of chestnut shoots *in vitro*. Physiol. Plant. 66, 491-494.

MEIJER E.A., DE VRIES S.C. & MORDHORST A.P. 1999. Co-culture with *Daucus carota* somatic embryos reveals high 2,4-D uptake and release rates of *Arabidopsis thaliana* cultured cells. Plant Cell Rep. 18, 656-663.

MICHALCZUK L., RIBNICKY D.M., COOKE T.J. & COHEN J.D. 1992 Regulation of indole-3-acetic acid biosynthetic path-ways in carrot cell-cultures. Plant Physiol. 100, 1346-1353.

MILLNER P.A. 2001. Heterotrimeric G-proteins in plant cell signalling. - New Phytol. 151: 165-174.

MINOCHA S.C. & NISSEN P. 1985 Uptake of 2,4-dichloro-phenoxyacetic acid and indole acetic acid in tuber slices of Jerusalem artichoke and potato. J. Plant Physiol. 120, 351-362.

MINOCHA S.C. 1987 pH of the medium and the growth and metabolism of cells in culture. pp. 125-141 in Bonga and Durzan (eds.) 1987 *Cell and Tissue Culture in Forestry* Vol 1. *General Principles and Biotechnology*. Martinus Nijhoff Publishers, Dordrecht, Boston, Lancaster. ISBN 90-247-3430-4.

MIYATA S., SUZUKI Y., KAMISAKA S. & MASUDA Y. 1981 Effects of abscisic acid on CO_2 exchange in *Lemna gibba*. Physiol. Plant. 51, 401-406.

MOCKAITIS K. & HOWELL S.H. 2000. Auxin induces mito-genic activated protein kinase (MAPK) activation in roots of *Arabidopsis* seedlings. Plant J. 24, 785-796.

MOK M.C. & MOK D.W. 1977 Genotypic responses to auxins in tissue culture of *Phaseolus*. Physiol. Plant. 40, 261-264.

MONTEUUIS O., BON M.C. & BERTHON J.Y. 1987 Micropropagation aspects of *Sequoiadendron giganteum* juvenile and mature clones. Acta Hort. 212, 489-497.

MORRIS D.A. 2000. Transmembrane auxin carrier systems – dynamic regulators of polar auxin transport . Plant Growth Regul. 32, 161-172,

MORRIS D.A., RUBERY P.H., JARMAN J. & SABATER M. 1991 Effects of inhibitors of protein synthesis on membrane auxin transport in *Cucurbita pepo* L. hypocotyl segments. J. Exp. Bot. 42, 773-783.

MORRIS D.A., FRIML J. & ZAZÍMALOVÁ E. 2004. The transport of auxins. pp.437-470.in Davies PJ (ed) *Plant Hormones: Biosynthesis, Signal Transduction, Action!* Springer/Kluwer Acad. Publ., N.Y., Dordrecht.
MUDAY G.K. 2000. Interactions between the actin cytoskeleton and an auxin transport protein. pp 541-556 in Staiger C.J, Baluska F, Volkmann D, Barlow P (eds.) *Actin: A dynamic framework for multiple plant cell functions*. Kluwer Academic Publishers, Dordrecht..
MUDAY G.K. & DELONG.A. 2001 Polar auxin transport: controlling where and how much. Trends Plant Sci 6, 535-542.
MURASHIGE T. 1961 Supression of shoot formation in cultured tobacco cells by gibberellic acid. Science 134, 280.
MURASHIGE T. & TUCKER D.P.H. 1969 Growth factor requirements of citrus tissue culture. pp. 1155-1161 in Chapman H. D. (ed.) Proc. 1st Int. Citrus Symp. Vol. 3, Univ. Calif., Riverside Publication.
NABORS M.W., HEYSER J.W., DYKES T.A. & DE MOTT K.J. 1983 Long-duration, high-frequency plant regeneration from cereal tissue cultures. Planta 157, 385-391.
NANDI S.K., TAMTA S. & PALNI L.M.S. 2002 Adventitious root formation in young shoots of *Cedrus deodara*. Biol. Plant. 45, 473-476.
NAPIER R.M., DAVID K.M. & PERROT-RECHENMANN C. 2002 A short history of auxin-binding proteins. Plant Mol. Biol. 49, 339-348.
NEGRUTIU I., JACOBS M. & GASPAR Th. 1979 Leaf formation and peroxidases from *Arabidopsis* callus. Z. Pflanzenphysiol. 91, 119-126.
NEMETH G. 1981 Adventitious root induction by substituted 2-chloro-3-phenyl-propionitriles in apple rootstocks cultured *in vitro*. Sci. Hort. 14, 253-259.
NEUMANN J. 1960 The nature of the growth-promoting action of coumarin. Physiol. Plant. 13, 328-341.
NEWCOMB W. & WETHERELL D.F. 1970 The effects of 2,4,6-trichlorophenoxyacetic acid on embryogenesis in wild carrot tissue cultures. Bot. Gaz. 131, 242-245.
NISSEN S.J. & SUTTER E.G. 1988 Stability of IAA and IBA in nutrient medium after autoclaving and after storage under various environmental conditions. HortSci. 23, 758.
MANLY J., SLOVIN J.P. & COHEN J.D. 2004. Auxin biosynthesis and metabolism. pp. 36-62 in Davies P.J. (ed): *Plant Hormones: Biosynthesis, Signal Transduction, Action*. Springer-/Kluwer Acad. Publ., New York, Dordrecht.
NORMANLY J., COHEN J.D. & FINK G.R. 1993 *Arabidopsis thaliana* auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. Proc. Natl. Acad. Sci. USA 90, 10355-10359.
NORMANLY J., GRISAFI P., FINK G.R. & BARTEL B. 1997 Arabidopsis mutants resistant to the auxin effects on indole-3-acetonitrile are defective in the nitrilase encoded by the NIT1 gene. Plant Cell 9, 1781-1790.
NORRIS R.F. & BUKOVAK M.J. 1972 Effect of pH on penetration of naphthaleneacetic acid and naphtheleneacetamide through isolated pea leaf cuticle. Plant Physiol. 49, 615-618.
NYMAN L.P. & ARDITTI J. 1984 Effects of 2,3,5-triodobenzoic acid on plantlet formation from cultured tissues of Taro, *Colocasia esculenta* L. Schott. (Araceae). Ann. Bot. 54, 459-466.
OKAMOTO I., ISOGAI, Y. & KOIZUMI, T. 1967. Isolation of indole-3- acetic acid, phenylacetic acid and several plant growth inhibitors from etiolated seedlings of *Phaseolus*. Chem. Pharm. Bull. 15: 159 - 163
OLIVER S.C., VENIS M.A., FREEDMAN R.B. & NAPIER R.M. 1995 Regulation of synthesis and turnover of maize ABP1 and observations of its passage to the plasma membrane: comparisons to BiP. Planta 197, 465-474.
PAL M. & NANDA K.K. 1981 Rooting of etiolated stem segments of *Populus robusta* – interaction of temperature, catechol and sucrose in the presence of IAA. Physiol. Plant. 53, 540-542.
PALME K., HESSE T., CAMPOS N., GARBERS C., YANOFSKY M.F. & SCHELL J. 1992 Molecular analysis of an auxin binding protein gene located on chromosome 4 of *Arabidopsis*. Plant Cell 4, 193-201.
PASTERNAK T.P., PRINSEN E., AYAYDIN F., MISKOLCZI P., POTTERS G., ASARD H., VAN ONCKELEN H.A., DUDITS D. & FEHER, A. 2002 The role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. Plant Physiology 129,1807-1819.
PAVLOVÁ L. & KREKULE J. 1984 Fluctuations of free IAA under inductive and non-inductive photoperiods in *Chenopodium rubrum*. Plant Growth Regul. 2, 94-98.
PERCIVAL F.W., PURVES W.K. & VICKERY L.E. 1973 Indole-3-ethanol oxidase. Kinetics, inhibition and regulation by auxins. Plant Physiol. 51, 739-743.
PHILLIPS D.J. 1961 Induction of a light requirement for the germination of lettuce seed by naringenin, and its removal by gibberellic acid. Nature 192, 240-241.
PHILLIPS D.J. 1962 Control of carnation streak virus by shoot tip culture. Phytopathol. 52, 747.
PLATT R.S. 1954 The inactivation of auxin in normal and tumorous tissue. Ann. Biol. 30, 349-359.
PONTIKIS C.A. & MELAS P. 1986 Micropropagation of *Ficus carica* L. HortSci. 21, 153.
PRZETAKIEWICZ A., ORCZYK W. & NADOLSKA-ORCZYK, A. 2003 The effect of auxin on plant regeneration of wheat, barley and triticale. Plant Cell Tissue Organ Cult. 73, 245-256.
PURVES W.K. & BROWN H.M. 1978 Indoleacetaldehyde in cucumber seedlings. Plant Physiol. 61, 104-106.
RAVEN J.A. 1975 Transport of indoleacetic acid in plant cells in relation to pH and electrical potential gradients, and its significance for polar IAA transport. New Phytol. 74, 163-174.
RAVEN J.A. 1979 The possible role of membrane electrophoresis in the polar transport of IAA and other solutes in plant tissues. New Phytol. 82, 285-291.
RAWAL S.K. & MEHTA A.R. 1982 Changes in enzyme activity and isoperoxidases in haploid tobacco callus during organogenesis. Plant Sci. Lett. 24, 67-77.
RAYLE D.L. & CLELAND R.E. 1992 The acid growth theory of auxin-induced cell elongation is alive and well. Plant Physiol. 99, 1271-1274
RAYLE D.L. & PURVES W.K. 1967 Conversion of indole-3-ethanol to indole-3-acetic acid in cucumber seedling shoots. Plant Physiol. 42, 1091-1093.
REDDY B.O., GIRIDHAR P. & RAVISHANKAR G.A. 2001 *In vitro* rooting of *Decalepis hamiltonii* Wight & Arn., an endangered shrub, by auxins and root-promoting agents. Curr. Sci. 81, 1479-1482.
REID J.B. & HOWELL S.H. 1995 The functioning of hormones in plant growth and development. pp. 448-485 in Davies, J.P. (ed.) *Plant Hormones, Physiology, Biology, Molecular Biology.* 2nd Ed., Kluwer Acad. Publ., Dordrecht, Boston, London.
REINECKE D.M. & BANDURSKI R.S. 1987 Auxin biosynthesis and metabolism. pp. 24-42 in Davies P.J. (ed.). *Plant Hormones and their Role in Plant Growth and Development.* Martinus Nijjhof, Boston.
RIBNICKY D.M., ILIC N., COHEN J.D. & COOKE T.J. 1996 The effects of exogenous auxins on endogenous indole-3-acetic acid metabolism – The implications for carrot somatic embryogenesis. Plant Physiol. 112, 549-558.
RUBERY P.H. & SHELDRAKE A.R. 1974 Carrier-mediated auxin transport. Planta 188, 101-121.

SACHS T. 2000 Integrating cellular and organismic aspects of vascular differentiation. Plant Cell Physiol 41, 649-656.
SAOTOME M., SHIRAHATA K., NISHIMURA, R., YAHABA M., KAWAGUCHI M., SYONO K., KITSUWA T., ISHII Y. & NAKAMURA T. 1993 The identification of indole-3-acetic acid and indole-3-acetamide in the hypocotyls of Japanese cherry. Plant Cell Physiol. 34, 157-159.
SARGENT J.A. & SKOOG F. 1960 Effects of indoleacetic acid and kinetin on scopoletin and scopolin levels in relation to growth of tobacco tissue *in vitro*. Plant Physiol. 35, 934-941.
SCHAEFFER G.W., BUTA J.G. & SHARPE F. 1967 Scopo-letin and polyphenol-induced lag in peroxidase catalyzed oxidation of indole-3-acetic acid. Physiol. Plant. 20, 342-347.
SCHERER G.F.E. & ARNOLD B. 1997 Inhibitors of animal phospholipase A(2) enzymes are selective inhibitors of auxin-dependent growth. Implications for auxin- induced signal transduction. Planta 202, 462-469.
SCHERER G.F.E. 2002 Secondary messengers and phospholipase A(2) in auxin signal transduction. Plant Mol. Biol. 49: 357-372.
SHARMA A.K., PRASAD R.N. & CHATURVEDI H.C. 1981 Clonal propagation of *Bougainvillea glabra* 'Magnifica' through shoot apex culture. Plant Cell Tiss. Organ Cult. 1, 33-38.
SHARP W.R., SONDAHL M.R., CALDAS L.S. & MARAFFA S.B. 1980 The physiology of *in vitro* asexual embryogenesis. Hort. Rev. 2, 268-310.
SIRIWARDANA S. & NABORS M.W. 1983 Tryptophan enhancement of somatic embryogenesis in rice. Plant Physiol. 73, 142-146.
SHELDRAKE A.R. 1973 The production of hormones in higher plants. Biol. Rev. 48 509-559.
SKOOG F. & MILLER C. 1957 Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. pp. 118-140 in Symp. Soc. Exptl. Biol., Number XI, The Biological Action of Growth Substances.
SKOOG F. & MONTALDI E. 1961 Auxin-kinetin interaction regulating the scopoletin and scopolin levels in tobacco tissue cultures. Proc. Natl. Acad. Sci. U.S.A. 47, 36-49.
SLOVIN J.P., BANDURSKI R.S. & COHEN J.D. 1999 Auxin. pp.115-140 in Hooykaas P.J.J., Hall M.A. & Libbenga K.R. (eds). *Biochemistry and Molecular Biology of Plant Hormones.* Elsevier Science B.V., Amsterdam.
SMITH S.M. & STREET H.E. 1974 The decline of embryogenic potential as callus and suspension cultures of carrot are serially subcultured. Ann. Bot. 38, 223-241.
SMULDERS M.J.M., JANSSEN G.F.E., CROES A.F., BARENDSE G.W.M. & WULLEMS G.J. 1988 Auxin regulation of flower bud formation in tobacco explants. J. Exp. Bot. 39, 451-459.
SNIR I. 1983 A micropropagation system for sour cherry. Scientia Hort. 19, 85-90.
SOBCZYKIEWICZ D. 1987 Mass propagation of raspberry plants by meristem culture. Acta Hort. 212, 607-609.
SOMERS D.A., GEGENBACH B.G., BIESBOER D.D., HACKETT W.P. & GREEN C.E. (ed.) 1986 Abstracts VI Int. Cong. Plant Tissue and Cell Culture. Internat. Assoc. Plant Tiss. Cult. Minneapolis, Minn.
STANGE L. 1979 Reversible blockage of the cell cycle in the meristem of *Riella helicophylla* (Bory et Mont.) Mont. by p-chlorophenoxyisobutyric acid (PCIB). Planta 145, 347-350.
STICKENS D., TAO W. & VERBELEN J.P. 1996 A single cell model system to study hormone signal transduction. Plant Growth Regul 18, 149-154.
STONIER T. & YONEDA Y. 1967 Stem internode elongation in the Japanese Morning Glory (*Pharbitis nil* Choisy) in relation to an inhibitor system of auxin destruction. Physiol. Plant. 20, 13-19.
STONIER T. 1969 Studies on auxin protectors. VII. Association of auxin protectors with crown gall development in sunflower stems. Plant Physiol. 44, 1169-1174.
STONIER T. 1971 The role of auxin protectors in autonomous growth. pp. 423-435 in *Les Cultures de Tissus de Plantes.* Coll. Int. C.N.R.S. No. 193, Paris.
STONIER T. 1972 The role of auxin protectors in autonomous growth. pp. 423-435 in *Les Cultures de Tissus de Plantes*. Proc. 2nd Int. Conf. Plant Tissue Cult. Strasbourg 1970.
STONIER T., HUDEK J., VANDE-STOUWE R. & YANG H.-M. 1970 Studies of auxin protectors. VIII. Evidence that auxin protectors act as cellular poisers. Physiol. Plant. 23, 775-783.
SUNDBERG B., LITTLE CH. A., CUI K. & SANDBERG, G. 1991 Level of endogenous indole-3-acetic-acid in the stem of *Pinus sylvestris* in relation to the seasonal-variation of cambial activity. Plant Cell Environ. 14, 241-246.
SUNDERLAND N. & WELLS B. 1968 Plastid structure and development in green callus tissues of *Oxalis dispar*. Ann. Bot. 32, 327-346.
SUNG Z.R. 1979 Relationship of indole-3-acetic acid and tryptophan concentrations in normal and 5-methyltryptophan-resistant cell lines of wild carrots. Planta 145, 339-345.
SYONO K. & FURUYA T. 1974 Induction of auxin-non-requiring tobacco calluses and its reversal by treatments with auxins. Plant & Cell Physiol. 15, 7-17.
SYONO K. 1979 Correlation between induction of auxin-nonrequiring tobacco calluses and increase in inhibitor(s) of IAA-destruction activity. Plant Cell Physiol. 20, 29-42.
TADINO V.L.A., FAEZ J.M., CHRISTIAENS L.E., KEVERS C., GASPAR T. & DOMMES, J. 2003 Synthesis and activity of another seleniated auxin: 2,4-dichlorophenylselenoacetic acid. Plant Growth Regul. 40, 197-200.
TAKAHASHI Y., ISHIDA S. & NAGATA T. 1995 Auxin-regulated genes. Plant Cell Physiol. 36, 383-390.
TE-CHATO S. & LIM M. 1999 Plant regeneration of mangosteen via nodular callus formation.- Plant Cell Tissue Organ Cult. 59, 89-93,
TERZI M. & LO SCHIAVO F. 1990 Developmental mutants in carrot. pp. 391-397 in Nijkamp H.I.J., Van Der Plas I.H.W. & Van Aartrijk J. (eds.) 1990 *Progress in Plant Cellular and Molecular Biology.* Proc. VIIth Int. Cong. on Plant Tissue and Cell Culture. Amsterdam, The Netherlands. 24-29 June 1990. Kluwer Academic Publishers, Dortrecht, Netherlands.
THEOLOGIS A. 1986 Rapid gene regulation by auxin. Annu. Rev. Plant Physiol. 37, 407-438.
THIMANN K.V. & MAHADEVAN S. 1964 Nitrilase. I. Occurrence, preparation and general properties of the enzyme. Arch. Biochem. Biophys. 105, 133-141.
THIMANN K.V. 1963 Plant growth substances, present and future. Annu. Rev. Plant Physiol. 14, 1-18.
THOMAS C., BRONNER R., MOLINIER J., PRINSEN E., VAN ONCKELEN H. & HAHNE G. 2002 Immuno-cytochemical localization of indole-3-acetic acid during induction of somatic embryogenesis in cultured sunflower embryos. Planta 215, 577-583.
THORPE T.A. & MURASHIGE T. 1968 Starch accum-ulation in shoot-forming tobacco callus cultures. Science 160, 421-422.
THORPE T.A. & MURASHIGE T. 1970 Some histo-chemical changes underlying shoot initiation in tobacco callus cultures. Can. J. Bot. 48, 277-285.

TIMPTE C. 2001. Auxin binding protein: curiouser and curiouser. Trends Plant Sci. 6, 586-590.

TIWARI S.B., HAGEN G. & GUILFOYLE TJ. 2004. Aux/IAA proteins contain a potent transcriptional repression domain. Plant Cell 16: 533-543.

TRYON K. 1956 Scopoletin in differentiating and non-differentiating cultured tobacco tissue. Science 123, 590.

TYBURSKI J. & TRETYN A. 2004 The role of light and polar auxin transport in root regeneration from hypocotyls of tomato seedling cuttings. Plant Growth Regul. 42, 39-48.

ULMASOV T., HAGEN G. & GUILFOYLE T.J. 1997a ARF1, a transcription factor that binds to auxin response elements. Science 276, 1865-1868.

ULMASOV T., LIU Z.-B., HAGEN G. & GUILFOYLE T.J. 1995 Composite structure of auxin response elements. Plant Cell 7, 1611-1623.

ULMASOV T., MURFETT J., HAGEN G. & GUILFOYLE T.J. 1997b Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9, 1963-1971.

VAN DER HOEF L. 1980 Auxin-regulated cell enlargement . - Is there action at the level of gene expression? pp. 159-175 in: Leaver C.J. (ed.) *Genome Organization and Expression in Plants,* NATO Advanced Study Institutes Series A, Vol. 29, Plenum Press, New York.

VAN DER KRIEKEN W.M., BRETELER H. & VISSER M.H.M. 1992 Uptake and metabolism of indolebutyric acid during root formation on *Malus* microcuttings. Acta Bot. Neerl. 41, 435-442.

VIDAL N., ARELLANO G., SAN-JOSE M.C., VIEITEZ A.M. & BALLESTER A. 2003 Developmental stages during the rooting of in-vitro-cultured *Quercus robur* shoots from material of juvenile and mature origin. Tree Physiol. 23, 1247-1254.

VOYIATZI C. & VOYIATZIS D.G. 1988 Shoot proliferation of the rose cv. (H.T.) Dr. Verhage as influenced by apical dominance regulating substances. Acta Hort. 226, 671-674.

VREUGDENHIL D., BURGERS A., HARKES P.A.A. & LIBBENGA K.R. 1981 Modulation of the number of membrane-bound auxin-binding sites during the growth of batch-cultured tobacco cells. Planta 152, 415-419.

WALDEN R. & LUBENOW H. 1996 Genetic dissection of auxin action: more questions than answers? Trends in Plant Sci. 1, 335-339.

WARD S.P. & ESTELLE M. 2001. Auxin signalling involves regulated protein degradation by the ubiquitin-proteasome pathway. J. Plant Growth Regul. 20, 265-273.

WEIS J.S. 1967 Auxin inactivation by normal and derived tobacco tissue cultures. Adv. Front. Plant Sci. 18, 155-161.

WELANDER M. & HUNTRIESER I. 1981 The rooting ability of shoots raised `*in vitro*' from the apple rootstock A2 in juvenile and in adult growth phase. Physiol. Plant. 53, 301-306.

WENT F.W. 1928 Wuchsstoff und Wachstum. Rec. Trav. Bot. Neerl. 25, 1-116.

WHITELEY E. & ABBOTT A.J. 1977 Microvegetative propagation of apples. Ann. Rep. Long Ashton Res. Sta. 1976, 62-63.

WIGHTMAN F. & COHEN J.D. 1968 Intermediate steps in the enzymatic conversion of tryptophan to IAA in cell. pp. 273-288. in Wightman F. & Setterfield G. (eds.) *Biochemistry and Physiology of Plant Growth Substances*. Runge Press, Ottawa.

WOO E.J., MARSHALL J., BAULY J., CHEN J.G., VENIS M., NAPIER R.M. & PICKERSGILL RW. 2002. Crystal structure of auxin-binding protein 1 in complex with auxin. EMBO J. 21, 2877-2885.

WOZNY A., GWODZ E. & SWEYKOWSKA A. 1973 The effect of 3- indoleacetic acid on the differentiation of plastids in callus culturs of *Cichorium intybus* L. Protoplasma 76, 109-114.

YAKUSHIJI H., MASE N. & SATO, Y. 2003 Adventitious bud formation and plantlet regeneration from leaves of fig (*Ficus carica* L.): J. Hort. Sci.Biotechnol. 78, 874-878.

Yang G.P., Bhuvaneswari T.V., Joseph C.M., King M.D. & Phillips, D.A. 2002 Roles for riboflavin in the *Sinorhizobium* – Alfalfa association.- Molecular Plant-Microbe-Interactions 15 (5): 456-462, 2002.

ZADOR E., KOVES E. & SZABO M. 1985 Phenolic materials in auxin heterotroph and autotroph (habituated) tobacco callus tissues. Biochem. Physiol. Pflanz. 180, 125-131.

ZAZIMALOVA E. & NAPIER R.M. 2003. Point of regulation for auxin action. Plant Cell Rep. 21: 625-634.

ZAZIMALOVA E., OPATRNY Z., BREZINOVA A. & EDER J. 1995 The effect of auxin starvation on the growth of auxin-dependent tobacco cell culture: dynamics of auxin-binding activity and endogenous free IAA content. J. Exp. Bot. 46, 1205-1213.

ZHANG C.L., CHEN D.F., ELLIOTT M.C. & SLATER A. 2004 Efficient procedures for callus induction and adventitious shoot organogenesis in sugar beet (*Beta vulgaris* L.) breeding lines. In Vitro Cell. Develop. Biol. 40, 475-481.

ZIMMERMAN R.H. & BROOME O.C. 1981 Phloroglucinol and *in vitro* rooting of apple cultivar cuttings. J. Am. Soc. Hort. Sci. 106, 648-652.

ZIMMERMAN R.H. 1984 Rooting apple cultivars *in vitro*: interactions among light, temperature phloroglucinol and auxin. Plant Cell Tiss. Organ Cult. 3, 301-311.

ZIMNY J. & LORZ H. 1986 Plant regeneration and initiation of cell suspensions from root-tip derived callus of *Oryza sativa* L. (rice). Plant Cell Rep. 5, 89-92.

Chapter 6
Plant Growth Regulators II: Cytokinins, their Analogues and Antagonists

1. BIOLOGICAL EFFECTS

Hormones in plants differ from most of those in animals by having pleiotropic effects; that is, they are involved in the control of a wide range of developmental processes. At the same time the effect of a hormone on any developmental process depends on the species. For example, ethylene inhibits growth in dicotyledons and most monocotyledons but is promotory in deepwater rice and other hydrophytes. Moreover, two or more hormones can interact synergistically or antagonistically in many circumstances. Equally, any given hormone may affect the biosynthesis or metabolism of another, thus affecting endogenous levels. The issue is further complicated by the fact that environmental factors - e.g. light, water status, wounding, pathogens - may modify responses and indeed hormone levels themselves. The reason for this appears to be that hormones (and growth regulators) and environmental factors share many components in their transduction chains (i.e. the very early events which occur after the signal - abiotic or biotic - is perceived by the plant tissue). These transduction chains interact to produce an integrated response.

Unsurprisingly therefore, it is difficult to predict how any hormone (or growth regulator or inhibitor) will affect any given plant system.

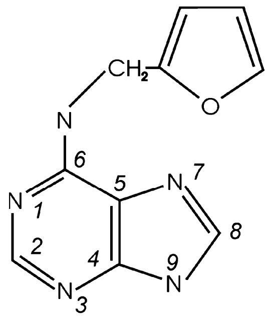
2. PROPERTIES AND DISCOVERY OF CYTOKININS

2.1. BIOLOGICAL ACTIVITY

Cytokinins comprise a separate class of growth substances and growth regulators. They produce various effects when applied to intact plants. They particularly stimulate protein synthesis and participate in cell cycle control. It is perhaps for this reason that they can promote the maturation of chloroplasts and delay the senescence of detached leaves. Cytokinin application to a single site in the plant (e.g. to one leaf) causes the treated organ to become an active sink for amino acids, which then migrate to the organ from surrounding sites. The effect of cytokinins is most noticeable in tissue cultures where they are used, often together with auxins, to stimulate cell division and control morphogenesis. Added to shoot culture media, these compounds overcome apical dominance and release lateral buds from dormancy.

2.2. DISCOVERY

As in the case of auxins, there are both naturally-occurring compounds and their synthetic analogues. The first cytokinin to be discovered, kinetin (1), was isolated in Professor Skoog's laboratory at the University of Wisconsin, following experiments to promote continuing growth of the callus which formed on tobacco stem sections on nutrient media. Cells of the explants initially proliferated quickly but although the addition of IAA increased the amount of callus produced, growth soon stopped and was not resumed even if pieces of the newly-formed tissue were subcultured onto a fresh medium. Cell division and callus growth did continue however, if either coconut milk or yeast extract were added to the medium and so attempts were made to isolate the active principle.



(1) kinetin

As chromatography of ethanol - soluble fractions of yeast extract indicated that the substance was a purine, other sources of naturally-occurring purines were examined for their ability to promote continued callus growth. Extracts from aged herring sperm DNA yielded a compound with the same adsorption peak and chemical behaviour as the one discovered from the yeast extract. It was also isolated in a crystalline form from samples of DNA autoclaved under acidic conditions. The new growth factor was named `kinetin' because it stimulated cell division in

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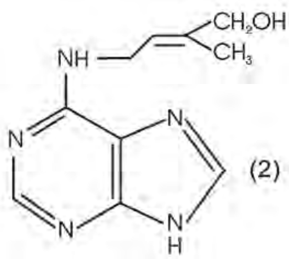
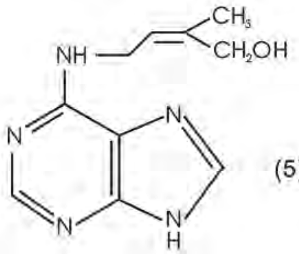
cells that otherwise might have become multinuclear (Miller *et al*., 1955a,b; Miller, 1961a,b). It was identified as 6-furfuryl aminopurine. The general term cytokinin was later proposed to cover all compounds having a similar activity (Skoog *et al*., 1965).

3. NATURALLY-OCCURRING CYTOKININS

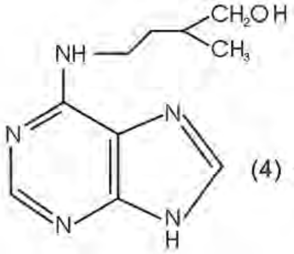
Although kinetin is not yet accepted as a naturally-occurring cytokinin, and thought to have arisen in the original isolates by structural rearrangement (Hecht, 1980), many natural cytokinins that are structurally-related to kinetin have been identified, either as free bases, as glucosides, ribosides, or nucleotides (Entsch *et al*., 1980). Such compounds used in plant tissue work are: *trans*-zeatin **(**2**)** (4-hydroxy-3-methyl-trans-2-butenylaminopurine), iP **(**3**)** (N^6 - Δ^2*iso*-pentenyladenine) and dihydrozeatin **(**4**)** **(**6-(4-hydroxy-3-methyl-trans-2- butenyl)aminopurine).



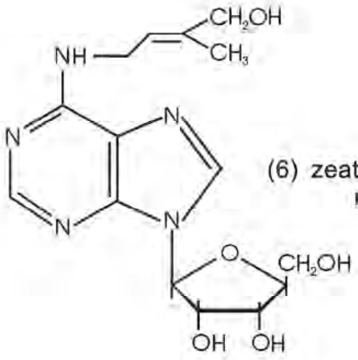
(2) *trans*-zeatin



(5) *cis*-zeatin



(4) *dihydro*-zeatin



(6) zeatin riboside

(3) iP (or 2-iP, or iPA)

Because of the presence of a double bond in the side chain, the zeatin molecule has two configurations: the form which occurs predominantly in nature is the *trans*-isomer. Large amounts of *cis*-zeatin (5) were, however, identified in *Cicer* seeds (Emery *et al*., 1998). Seaweed preparations are today widely used to modify plant growth both in the field and under *in vitro* situations. Recent work has shown that seaweeds are rich sources of natural *cis*-zeatin (Stirk *et al*., 2003). These results indicate that the *cis*-isomer is of much wider occurrence and significance than originally thought. Synthetic preparations of zeatin often consist of mixed *cis*- and *trans*-isomers but the *cis*- form has much lower cytokinin activity (Van Staden and Drewes, 1991). This low activity of zeatin-*cis*-isomers can be explained in terms of the existence of *cis-trans*-isomerase (Mok *et al*., 1992; Bassil *et al,* 1993) having high affinity to convert *cis*-zeatin to the highly active *trans*-isomer. Besides occurring as a free base, zeatin also occurs naturally as glucosidic conjugates. Dihydrozeatin, a natural metabolite of zeatin, has cytokinin activity; it is resistant to oxidation due to its saturated side chain, and is sometimes considered as a storage form of zeatin. Zeatin riboside (6) is highly active as a cytokinin, and has been used in some experimental

tissue culture work. The riboside of iP [N^6-(Δ^2-isopentenyl)adenosine], or its 2-methylthio-analogue, can occur in t-RNAs (see below). In *Actinidia* cultures, iP is converted to zeatin (Einset, 1986a,b).

Despite the occurrence of endogenous cytokinins in whole plants, many tissues and small organs isolated *in vitro* are unable to synthesize sufficient of these substances to sustain growth. This is particularly the case with dicotyledonous tissues where a low level of cytokinin is frequently required to be added to the culture medium. The dependence of some callus cultures on cytokinin for cell division has been used as the basis of a sensitive bio-assay for cytokinins (Miller, 1963; Linsmaier and Skoog, 1965; Letham, 1967). However, cytokinin-independent callus and cell strains of broad-leafed plants are commonly found, and from some of them, zeatin and iP have been isolated (Dyson and Hall, 1972; Einset and Skoog, 1973). Zeatin and zeatin riboside have been obtained from monocotyledonous callus grown in the absence of either auxin or cytokinin (Kemp and Stoltz, 1979). Presumably in tissues that are able to grow without cytokinin being added to the medium, the cells can produce sufficient natural cytokinin for cell division to proceed.

3.1 BIOSYNTHESIS

Cytokinins occur as free molecules in plants, but are also found in the t-RNAs of the cytoplasm and chloroplast. In whole plants, roots appear to be major sites of natural cytokinin biosynthesis, but some production does take place in other actively growing tissues, (Van Staden and Davey, 1979; Chen *et al*., 1985). The root apex, and particularly the cells of its 'quiescent centre', could be important sites of synthesis (Short and Torrey, 1972a; Torrey, 1972). It has been suggested that the slow rate of cell division in the root quiescent centre could be the result of a supra-optimal cytokinin concentration. Some experimental evidence that the root tip is a primary site of cytokinin synthesis was obtained by Ochatt and Power (1988) in the roots which developed from the callus of *Prunus cerasus*. Roots only differentiated some distance from the tip, leaving a lateral-root-free zone, but were formed along the entire length of the root, right up to the tip when the root tip was removed. It was thought that cytokinins might have been present in supra-optimal amounts for rooting, until the tip was removed.

Isolated roots can be cultured without the addition of a cytokinin to the growth medium. Skoog and Tsui (1948) found that although roots, initiated from tobacco calluses, were able to grow indefinitely in White's basic medium without growth substances, shoot buds were incapable of continued growth unless they developed either directly from roots, or from callus immediately adjoining the origin of the adventitious roots. Similar observations have been made by Koda and Okazawa (1980) with asparagus, and by Bollmark *et al*. (1988) with pea cuttings.

CK biosynthesis has been dogged by unproven assumptions and mis-conceptions since these compounds were first discovered in plants. As a result of the circumstances that led to their discovery and because of their impact on both cell division and protein synthesis, CKs were always thought to be closely associated with nucleic acids. For over a decade efforts focussed solely on the possibility that CKs were derived from t-RNA and it was only in the late 1970s that a direct route of CK biosynthesis, independent of t-RNA, was proposed. However, many of the original ideas concerning CK biosynthesis continued to plague this branch of CK research. Whilst significant advances have been made in the last three years, there is still much to be learnt, particularly concerning the nature of the substrates for the key enzyme for CK biosynthesis, i.e. isopentenyl transferase (IPT) (see Fig. 6.1).

The continued use of genomics integrated with traditional biochemical and genetic approaches should allow the elucidation of the 'elusive' isoprenoid CK biosynthetic pathway. However, it must be emphasised that the use of any of these techniques alone will be insufficient and result in an incomplete picture of CK biosynthesis. It is of prime importance to establish the universality of the *IPT* gene in plants, which should become increasingly easier as more plant genomes are mapped, and by applying techniques similar to those used by Zubko *et al.* (2002) and Sun *et al.,* (2003). Of equal importance is the determination of the origin of the side chain and the nature of the reaction products. By utilising the methods of Åstot *et al*. (2000) in plants where native genes are overexpressed, together with an inhibitor of the methylerythritol phosphate (MEP) pathway for formation of dimethylallyl diphosphate (DMAPP), one of the substrates for IPT, this area of uncertainty should be resolved. The recently discovered predominance of zeatin *cis*-isomers in certain plant species also requires explanation and relates to the possibility that these *cis*-isomers may be synthesised via a direct route, independent of t-RNA degradation. Finally, the most ignored area of CK biosynthesis deserves some attention, i.e. the

biosynthesis of the aromatic CKs. These derivatives presumably play an important role in many plant species, yet research into this species of CK is minimal at the most. A starting point would be to examine the incorporation of label from previously proposed side chain precursors in cell free extracts, as was achieved with isoprenoic CKs, and possibly to identify mutants in which aromatic CKs are over-produced (Taylor *et al.*, 2003).

One way in which the origin of the side chain could be resolved would be to over-express key genes involved in the MEP and acetate/mevalonate (MVA) pathways in callus tissue and then to assess whether shoot formation occurs in the absence of exogenously applied CK as achieved by Kakimoto (2001) and Sun *et al.* (2003), and then to determine the CK profile by GC-MS (Taylor *et al.*, 2003). A similar strategy has been employed in *Arabidopsis* to ascertain if GA biosynthesis is plastid-localised (Estévez *et al.*, 2001). Alternatively, chloroplasts could be isolated and fed labelled pyruvate or GA-3-P and CKs analysed to assess if any have incorporated label. This could be confirmed by feeding label in the presence of fos-midomycin, an inhibitor of the plastid biosynthetic pathway, which should result in a decrease in incorporation into CKs. Of utmost importance is the unequivocal demonstration of incorporation of label from the MEP or acetate/MVA pathways into CKs.

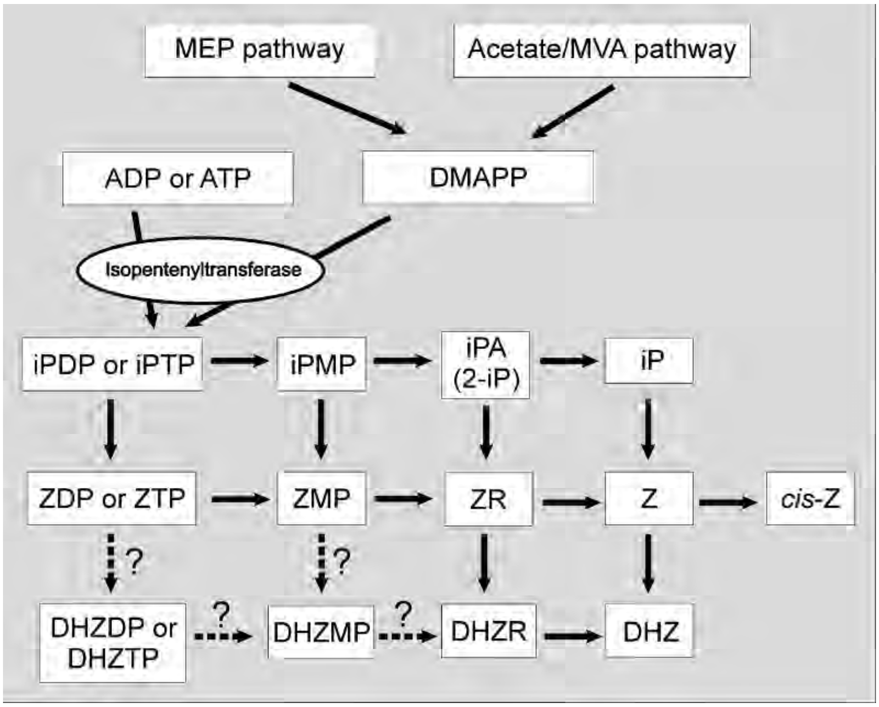


Fig. 6.1 Scheme of cytokinin biosynthesis in plants (according to Buchanan *et al.*, 2000, and Haberer and Kieber, 2002, modified). MEP, methylerythritol phosphate; MVA, mevalonic acid; DMAPP, dimethylallyl diphosphate; iPDP, N^6-(Δ^2-isopentenyl)adenosine-5′-diphosphate; iPTP, N^6-(Δ^2-isopentenyl)adenosine-5′-triphosphate; iPMP, N^6-(Δ^2-isopentenyl)adenosine-5′-monophosphate; iPA, N^6-(Δ^2-isopentenyl)adenosine; iP, N^6-(Δ^2-isopentenyl)adenine; Z, zeatin; ZDP, zeatin riboside-5′-diphosphate; ZTP, zeatin riboside-5′-triphosphate; ZMP, zeatin riboside-5′-monophosphate; ZR, zeatin riboside; *cis*-Z, *cis*-zeatin; DHZDP, dihydrozeatin riboside-5′-diphosphate; DHZTP, dihydrozeatin riboside-5′-triphosphate; DHZMP, dihydrozeatin riboside-5′-monophosphate; DHZR, dihydrozeatin riboside; DHZ, dihydrozeatin.

Cytokinins produced in roots of plants are normally transported in the xylem to other regions. The bleeding sap of plants is rich in cytokinins and has been shown to promote growth *in vitro* (Skene, 1972b; Zimmer and Pieper, 1975; 1976). The cytokinin produced by shoot tissues is only a small proportion of that formed by root apices. That synthesised in shoot apices is insufficient to sustain their prolonged growth *in vitro*.

Cytokinin nucleotides (compounds composed of D-ribose, phosphoric acid, and N^6-substituted adenine) are characteristic of natural cytokinins. They are found in suspension cultured cells, but whether they arise from the degradation of t-RNA or from *de novo*

synthesis, is not clear. That they might be a source of cytokinins in cultures is suggested by the finding that their maximum concentration in *Pimpinella anisum* suspensions coincided with the peak occurrence of isopentenyladenine, and iso-pentenyladenosine in the cells (Ernst and Oesterholt, 1985).

4. SYNTHETIC CYTOKININ ANALOGUES

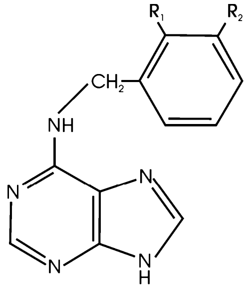
4.1. SUBSTITUTED PURINES

Although used in research, the natural cytokinins iP and zeatin are not used by commercial laboratories routinely, because of their cost. Fortunately, several chemical analogues of natural cytokinins apart from kinetin have been prepared which are found to be highly active as cytokinins. Although they are chiefly N^6-substituted adenine derivatives, some other slightly less structurally-related compounds also possess cytokinin activity, for example 4-alkylaminopteridines (Iwamura *et al.*, 1980), and 6-benzyloxypurines. Some of these analogues are reported to be more active than kinetin or benzyladenine (BA), and are particularly effective in promoting morphogenesis (Wilcox *et al.*, 1978, 1981). The 1-deaza analogue of zeatin riboside (Rogozinska *et al.*, 1973; Rodaway and Lutz, 1985; Kaminek *et al.*, 1987) also has cytokinin activity. Today it is widely accepted that derivatives of BA (7a), such as the topolins (7b,c) are aromatic, naturally occurring cytokinins. It is essential that they be treated as such in tissue culture research and experimental design.

Only a few of these compounds are available in chemical catalogues. They are expensive and therefore only of interest for research purposes. Synthetic cytokinins most commonly used in micropropagation work are the compounds kinetin (1) and benzylaminopurine (BA) (7a).

It is now accepted that BA-derivatives e.g 2-hydroxy benzyladenosine (8) are naturally occurring (Horgan *et al.*, 1973; Ernst *et al.*, 1983). The synthetic cytokinin PBA (or SD8339) (9) 6-(benzyl-amino)-9-(2- tetrahydropyranyl)-9H-purine, which is a product of Shell Research Ltd., has high physiological activity, but seems only to have been used experimentally, and not in commercial micropropagation.

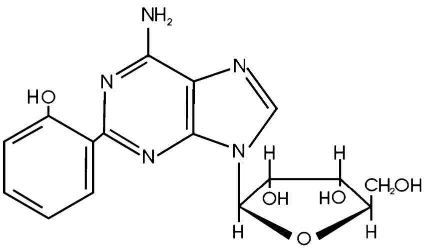
Several compounds with cytokinin or anticytokinin activity have fungicidal properties (Hecht, 1980). The fungicide benomyl (10), which has a structure broadly similar to adenine-based cytokinins, has the ability to stimulate the growth of soybean and radish callus cultures (Skene, 1972a). In both species it was much less efficient than kinetin. Benomyl can be damaging to cultures. It was phytotoxic to orchid protocorms at 0.2 mg/l in an agar medium, but stimulated growth at lower levels (Gupta and Hadley, 1977).



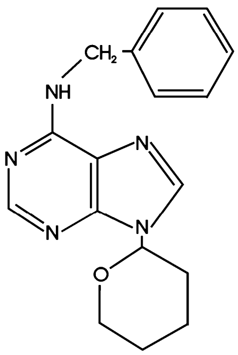
(7a) $R_1 = R_2 = H$
BA [N^6-benzyladenine]

(7b) $R_1 = OH$, $R_2 = H$
ortho-topolin [N^6-(2-hydroxybenzyl)adenine]

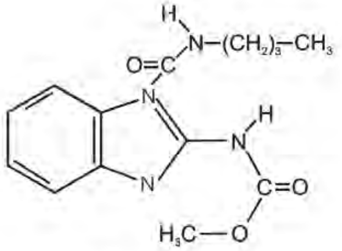
(7c) $R_1 = H$, $R_2 = OH$
meta-topolin [N^6-(3-hydroxybenzyl)adenine]



(8) 2-hydroxybenzyladenosine



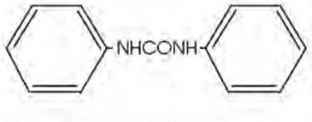
(9) PBA (SD8339)



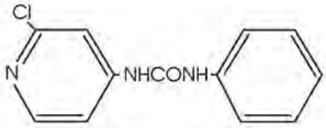
(10) benomyl

4.2. THE PHENYLUREAS

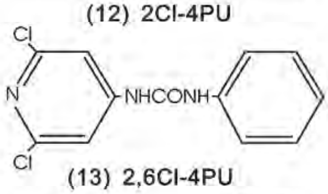
A common way of adding a natural cytokinin to media is by the use of organic supplements such as yeast extract or coconut milk. Coconut milk contains several physiologically-active substances (see Chapter 4); amongst which have been found the natural cytokinin zeatin, and 1,3-diphenylurea (11) (Shantz and Steward, 1955). The latter compound, and many other substituted ureas, have cytokinin activity and can promote the growth of dormant buds (Kefford *et al*., 1966), and induce cell division in cytokinin-dependent callus tissues (Bruce *et al*. 1965; Bruce and Zwar, 1966). Diphenylureas are not commonly used in tissue cultures, but there are a few reports. Butenko *et al*. (1972) found 2 mg/l 1,3-diphenylurea facilitated organogenesis in sugar beet callus cultures. Some *N*-pyridyl-*N′*-phenylureas are more active than N^6-substituted purines such as BA and zeatin in promoting callus growth and morphogenesis in tobacco and several other kinds of plants (Okamoto *et al*., 1978; Takahashi *et al*., 1978; Kamada and Harada, 1979; Ohyama and Oka, 1982). Two of the most active of the compounds in this series are 2Cl-4PU **(**12**)** (or CPPU) *N*-(2-chloro-4-pyridyl)-N′- phenylurea, and 2,6Cl-4PU **(**13**)** **(** *N*-(2,6-dichloro-4-pyridyl)-N′-phenylurea). The thiadiazole-substituted phenylurea: thidiazuron (TDZ) (14) (N-phenyl-N′-1,2,3-thiadiazol-5-ylurea) which was registered as a cotton defoliant (Arndt *et al*., 1976) and given the product name 'Dropp', has high cytokinin activity (Mok *et al*., 1982). In some plants, it is more effective than adenine-based compounds for inducing adventitious shoot regeneration (e.g. indirectly in *Vitis vinifera* cvs. - Reisch and Martens, 1988; and directly in *Rhododendron* - Imel and Preece, 1988, and *Malus* - Elobeidy and Korban, 1988). TDZ can also be highly effective for inducing axillary shoot formation in shoot cultures (e.g. in *Rorippa* - Gilby and Wainwright, 1989; *Rhododendron* - Fellman *et al*., 1987; and *Malus domestica* - Fasolo *et al*, 1988a, b). Although many shoots can be formed, they may not elongate sufficiently.



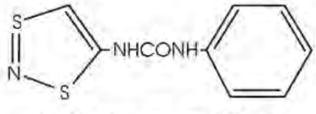
(11) 1,3 - diphenylurea



(12) 2Cl-4PU



(13) 2,6Cl-4PU



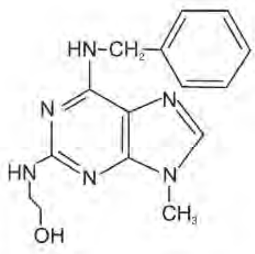
(14) thidiazuron (TDZ)

Effective shoot proliferation in the otherwise difficult-to-propagate *Acer fremanii* (Kerns and Meyer, 1986), and in *Pyrus communis* (Singha and Bhatia, 1988), was achieved using mixtures of BA and TDZ (respectively 1 μM + 0.01 μM for *Acer* and 5 μM + 0.4 mM for *Pyrus*). In both cases there was a significant interaction between the two compounds. Fellman *et al*. (1987) also found that 0.5 μM 2Cl-4PU was effective for inducing *Rhododendron* shoot proliferation: shoot elongation was inhibited at higher levels.

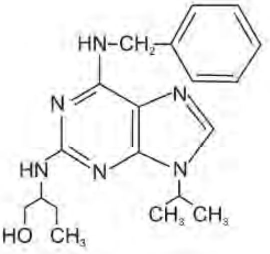
5. MODE OF ACTION

Cytokinins play multiple roles in the control of plant development; however, the mode of their action at the molecular level is uncertain. Some are present in t-RNA molecules, but it is not yet clear whether incorporation into t-RNA is necessary before typical cytokinin effects can become apparent. In some circumstances, cytokinins activate RNA synthesis, stimulate protein synthesis and the activities of some enzymes (Kulaeva, 1980). Cytokinin treatment also results in an increase of the polyribosome content in

cultured soybean cells (Tepfer and Fosket, 1978). Although some workers have recorded a low level of incorporation of a synthetic cytokinin analogue into t-RNA (Burrows *et al.*, 1971; Peaud-Lenoel and Jouanneau, 1980), this could not be correlated with the observed physiological action of the compound.



(15) olomoucine



(16) roscovitine

(17) bohemine

The action of cytokinins is light-dependent. In blue, far-red and white light, the proliferation of shoots of *Prunus* by BA was strongly dependent on the rate of photon fluence, but the rate of fluence of a red light source was not critical. Baraldi *et al.* (1988) suggested that shoot proliferation by this cytokinin was promoted by a low energy phytochrome response. In conditions which do not induce shoot proliferation, viz. dark, or low fluence far-red light, BA inhibits shoot elongation. Promotion of axillary shoot growth by BA, and its inhibition of shoot elongation, therefore seem to be two independent processes.

Cytokinins, together with auxins, take part in the regulation of the cell cycle in plant cells. They probably induce D-type cyclin CycD3 and thus stimulate the cell cycle progression from the G1 to the S phase, and possibly also G2/M transition via induction of expression of the gene *CDC2* for histon-H1-kinase and stimulation of its dephosphorylation by Cdc25 (reviews by John *et al.,* 1993, Frank and Schmülling, 1999, Pasternak *et al.*, 2000; den Boer and Murray, 2000). Interestingly, C^2-, N^6-, and N^9-trisubstituted derivatives of cytokinins (olomoucine (15), roscovitine (16) and bohemine (17) are able to inhibit cyclin-dependent kinases of type 1 and 2 in both human and plant cells and consequently are able to block the cell cycle in the G1/S and G2/M transitions (Vesely *et al.*, 1994; De Azevedo *et al.*, 1997; Havlicek *et al.*, 1997; Binarova *et al.*, 1998; Planchais *et al.*, 2000).

Recently, some histidine kinases, homologous to bacterial two-component sensor kinases, have been identified as candidates for cytokinin receptors. This finding implies that the downstream signalling cascade is similar to the phosphorelay mechanism seen with ethylene receptors (see Chapter 7). The main representatives of possible CK receptors are CKI1 (Kakimoto, 1996) and CRE1/AHK4/WOL (Mähonen *et al.*, 2000; Inoue *et al.*, 2001; Suzuki *et al.*, 2001). After perception of the cytokinin signal the downstream cascade begins by the phospho-transfer between histidine and aspartate residues within the kinase, resulting then in the phosphorylation of AHP proteins. These activated AHPs are translocated from the cytosol into the nucleus where they activate so-called B-type ARR proteins, leading to derepression of target genes involved in regulation of cell division, shoot formation and delay of senescence. Activated B-type ARR proteins also increase the transcription of A-type ARRs which provide a feed-back control of the whole system (reviewed by Hwang and Sheen, 2001; Deruere and Kieber, 2002; Haberer and Kieber, 2002; Schmülling, 2002). The recent discoveries of candidate CK receptors of the histidine kinase type and downstream elements of a CK signalling pathway represent a real breakthrough in our understanding of the molecular mechanism of cytokinin signalling. However, the final output of the signalling cascade, starting at the plasma membrane and leading to regulation of gene expression, is still unclear.

5.1. ANTI-AUXIN EFFECT

Some aminopurines with cytokinin properties act as reductants in a photochemical reaction with riboflavin, and are oxidised thereby to adenine (Rothwell and Wright, 1967). Part of the biological effects produced by cytokinins could be due to their inhibition of the oxidation of IAA. Kinetin (0.04-1 mg/l) alters the activity, distribution, and composition of IAA oxidase isoenzymes within tobacco callus cells (Lee, 1974).

Noting that callus which was induced to form shoots by the addition of cytokinin was more compact than non-shoot-forming callus, Kirkham and Holder (1981) investigated the effect of kinetin on callus water potential; the cytokinin made cell walls

more rigid, so that the turgor potential of cells was increased. The water potential of the cells was therefore increased (made less negative), and they became less liable to take up water from the surrounding medium. This was directly opposite to the effect of the auxin PCPA.

5.2. CARBOHYDRATE METABOLISM

Apart from a possible effect on levels of endogenous auxin, cytokinins appear to be implicated in sugar metabolism. Both decreases and increases in the specific activity of enzymes of the glycolytic and oxidative pentose phosphate pathways have been reported. A medium containing adenine sulphate in addition to kinetin, which is conducive to shoot formation in *Nicotiana tabacum*, was noted by Scott *et al*. (1964) to cause a marked increase in the activities of two enzymes of the oxidative pentose phosphate pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), compared to their activities in a non-shoot-forming medium. Conditions favouring bud formation, including the availability of cytokinins, seem to enhance starch metabolism in tobacco callus (Thorpe and Meier, 1972). Callus, which produces shoots had high specific activities of enzymes involved in both starch accumulation and breakdown (Thorpe and Meier, 1974). Cytokinins reduce the oxygen uptake of cells (Neumann, 1968) and inhibit the alternative cyanide-resistant respiration pathway, which exists in many plants (Miller, 1979; 1980; 1982: Musgrave and Siedow, 1985).

5.3. THE PHENYLUREA COMPOUNDS

It is generally assumed that phenyl urea compounds with cytokinin activity act at the same sites as the purine-based cytokinins, i.e. that they are active in their own right. This hypothesis was put forward by Kurosaki *et al*. (1981), who suggested that there were structural similarities between the two classes of compound. It was further supported by computer modelling of possible interactions between active cytokinins and putative cytokinin receptor molecules (Fox, 1992). An alternative hypothesis is that the phenylureas may stimulate the accumulation, or biosynthesis, of natural purine-based cytokinins, or alter the metabolism of these compounds (see below). The phenylureas are potent inhibitors of cytokinin oxidase (Horgan, 1987a).

The activity of thidiazuron as a defoliant seems to be associated with its capacity to induce ethylene production in treated leaves (Suttle, 1984). Thidiazuron-induced leaf drop can be inhibited by AVG (see Chapter 7), which prevents ethylene biosynthesis (Elstner *et al*., 1983), or treatments which prevent ethylene action (Suttle, 1985). How this relates to the marked cytokinin activity of the compound is unclear but may relate to the effects of cytokinins on ethylene biosynthesis (See Chapter 7).

6. UPTAKE AND METABOLISM

The uptake of cytokinins into cultured tissues is rapid (Marino, 1986; Mariano, 1988). The metabolism of cytokinins is rather complex and generally consists of conversions among cytokinin bases, ribosides and ribotides, and conjugation and degradation reactions (Van Staden and Crouch, 1996; Zazimalová *et al*., 1999).

A naturally-occurring enzyme, cytokinin oxidase, degrades cytokinins such as zeatin and isopentenyladenine which have a Δ^2-double bond, by cleaving the side chain (Fig. 6.2) (Horgan, 1987a; Chatfield and Armstrong, 1988); cytokinins with saturated (dihydrozeatin) and/or bulky (aromatic cytokinins, *o*-glucosides) side-chains, as well as cytokinin nucleotides, are not substrates for this enzyme. Rapid degradation of zeatin and iP by the enzyme could explain the ineffectiveness of these compounds on plants, such as *Gerbera*. In several different kinds of plant tissue, the activity of cytokinin oxidase is enhanced by exogenous application of cytokinins (Palmer and Palni, 1987; Motyka and Kaminek, 1990) suggesting that treating plants with synthetic cytokinins could decrease the level of the natural endogenous compounds. In some other reports treating plant tissues with synthetic homologues caused at least a temporary increase in levels of natural zeatin, and zeatin riboside (Thomas and Katterman, 1986; Hansen *et al*., 1987; Vankova *et al*., 1992). In *ipt* gene-transformed tobacco plants (coding for isopentenyltransferase - the 'front' enzyme of cytokinin biosynthesis in *Agrobacterium*) the derepression of the gene leads to an increase of endogenous cytokinins, which is immediately followed by an increase in cytokinin oxidase activity (Motyka *et al*., 1996). This finding supports the hypothesis that cytokinin oxidase is a substrate-inducible enzyme, which plays an important role in maintenance of cytokinin homeostasis in plant cells.

In some plants, an enzyme other than cytokinin oxidase is thought to be responsible for the degradation of kinetin and benzyladenine to adenine (Forsyth and Van Staden, 1987); a considerable amount of side chain cleavage was found in the shoots from *Gerbera* shoot cultures (Blakesley and Lenton, 1987). In other plants BA is not broken down in this way. In *Gerbera* callus virtually no side chain cleavage was detected after 90 h of culture with BA (Blakesley and Lenton, loc. cit.), and adenine or adenosine were not produced from BA in soybean callus (Van Staden and Mooney, 1988). In *Prunus domestica* shoot cultures, at least some of the applied BA is broken down to CO_2 over 21 days (Mariano, 1988).

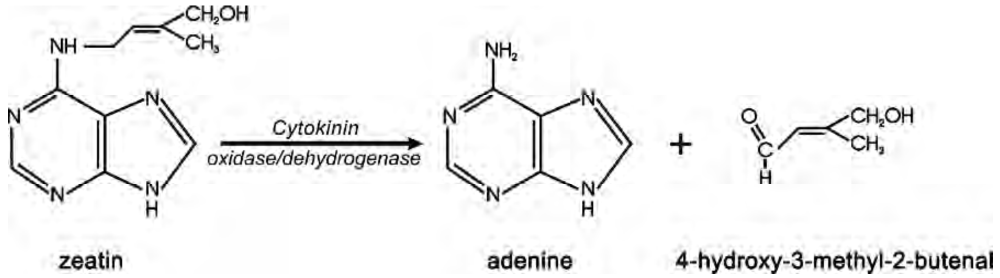


Fig. 6.2 Cytokinin degradation.

In *Gerbera* callus, free zeatin becomes conjugated with ribose or ribose phosphate within 10 h to produce the physiogically-active compounds, zeatin-9-riboside and zeatin-9-ribotide, but these conjugates then disappear during the next 30h and adenosine and other products of side- chain cleavage accumulate (Blakesley and Lenton, 1987).

In various plants, BA is initially converted to several metabolites, which are probably, mono-, di- and tri-ribotides, the 3-, 7- and 9-glucosides, a 9-riboside, a 9-ribotide, and a 9-riboside-glucoside (Blakesley and Lenton, 1987; Horgan, 1987b; Van der Krieken *et al*., 1988). The formation of these conjugates does not necessarily mean that BA is inactivated, as one or more of the conjugates may still have cytokinin activity (Van der Krieken *et al*., 1988; Van Staden and Drewes, 1992), or may act as storage products.

7. EFFECTS IN TISSUE CULTURES AND PLANT ORGANS

7.1. STIMULATION OF CELL DIVISION

In tissue cultures (as well as in intact plants and plant organs), cytokinins appear to be necessary for plant cell division (cf. 'Mode of Action' above). In their absence, metaphase, but not prophase of mitosis, is considerably protracted, and it has been suggested that cytokinins might be required to regulate the synthesis of proteins involved in the formation and function of the mitotic spindle apparatus (Jouanneau, 1970, 1975). In cultures where cytokinin is limiting, division of cell nuclei becomes arrested at one stage of the cell cycle. Subculture of the tissue onto a medium containing a cytokinin can then cause the cells to divide synchronously after a lag period (Jouanneau, 1971). Callus tissues in which cell division proceeds without the addition of cytokinin to the culture medium (e.g. *Oxalis dispar* - Sunderland and Wells, 1968), are thought to be able to produce their own natural growth substances. Three natural cytokinins could be isolated, for example, from a cytokinin-independent strain of tobacco callus (Skoog *et al*., 1973). In semi-synchronised cytokinin-independent tobacco cell suspension culture the individual onset of cell division during the exponential growth phase correlated with peaks of endogenous iP, zeatin and their ribosides (Zazimalová *et al*., 1996). In synchronised BY-2 tobacco cell suspension the maximum of endogenous cytokinins (largely zeatin) preceded G2/M transition (Redig *et al*., 1996, Laureys *et al*., 1998)

Callus proliferation from the tissues of most dicotyledonous plants is usually thought to require the presence of both an auxin and a cytokinin in the growth medium, but Nitsch and Bui-Dang-Ha (1967) found that proliferation of tobacco pith explants would take place if a synthetic cytokinin and an auxin were supplied sequentially in that order. Thus, when grown for one day on a basal medium containing 0.2 mg/l kinetin, before being transferred for 20 days to the same basal medium but with 1.8 mg/l IAA, more callus was produced than when the same quantities of auxin and cytokinin were available together. By

contrast, there was practically no growth at all if IAA was provided for only one day, and kinetin for the rest of the time. The sequential promotion of growth was not apparent however, when natural cytokinins were employed. Nitsch (1968) interpreted these results to indicate that cytokinins act during the pre-treatment phase at the DNA level (even though no cell divisions occur during this period) and that natural cytokinins were degraded too rapidly to be effective, except when present in the medium continuously. Direct mutual regulatory effects between auxin and cytokinin was observed at *Nicotiana plumbaginifolia* at the gene level (Dominov *et al*., 1992).

Dicotyledonous callus, or suspension cultures requiring auxin (e.g. 1 mg/l IAA) but not cytokinin for growth, can be cultured for long periods without auxin when a high concentration of cytokinin (e.g. 0.1-1 mg/l kinetin) is added to the medium. At this level the cytokinin appears to increase the natural auxin content of the tissues but not to cause auxin-habituation, because after a prolonged period of culture, removal of the cytokinin again causes the cells to become auxin-dependent (Syono and Furuya, 1972). In transformed tissue the expression of the gene coding for iso-pentenyltransferase resulted in an increase of endogenous cytokinins and a parallel decrease of endogenous IAA (Akiyoshi *et al*., 1983); similarly, the application of synthetic auxin NAA led to a decrease of endogenous iP and zeatin in tobacco cells (Vanková *et al*. 1992). In contrast to this, reduction of the auxin concentration in the cultivation medium resulted in a very significant increase of endogenous cytokinins, namely iP and zeatin (Zazimalová *et al*., 1996). It is apparent that not only auxins and cytokinins *per se*, but the levels of both hormones and particularly the proportion of one to the other (cf. Skoog-Miller model of plant morphogenesis, 1957, see auxin-cytokinin interaction, below) are determinants for cell cycle, cell division and differentiation control.

7.2. ADVENTITIOUS SHOOT FORMATION

Cytokinins are very effective in promoting direct or indirect shoot initiation. As mentioned in the previous section, they are used for this purpose in combination with auxins. A balance between auxin and cytokinin normally gives the most effective organogenesis.

Leaf segments of *Crassula argentea* form callus in response to wounding, which then gives rise to roots and later, shoots. Paterson and Rost (1981) found that if cytokinin was added to the medium, shoots were formed from a superficial meristem, and roots were afterwards produced from inside the callus. The normal sequence of organogenesis was therefore reversed.

7.3. EMBRYOGENESIS

A low concentration of cytokinin (typically 0.5-2.5 μM) is often added to media for the induction of embryogenic callus, especially in broad-leafed plants (see Chapter 10). There is, however, some evidence to suggest that cytokinins may inhibit embryogenesis in monocotyledons: 0.001 μM exogenous cytokinin was sufficient to prevent it in *Dactylis glomerata.* The presence of endogenous cytokinin may also be responsible for the inability to obtain embryogenesis in some genotypes. Leaf sections of non- embryogenic strains of this grass contained less natural cytokinin than those which were capable of producing embryogenic callus (Wenck *et al*., 1988). Carman and Campbell (1988) were able to induce embryogenesis in a non-responsive strain of wheat by detaching wheat spikes from the plant some while before culturing immature embryo explants. This was thought to decrease the supply of natural cytokinins from the roots. If zeatin was added to the medium, embryogenesis was suppressed.

7.4. USE IN SHOOT CULTURES

7.4.1. Axillary shoot proliferation

To encourage the growth of axillary buds, and reduce apical dominance in shoot cultures of broad-leafed plants, one or more cytokinins are usually incorporated into the medium at Stage II. A successful treatment induces the growth of several small shoots from each explant over a 4-6 week period. Levels of cytokinin, which are too high, cause many small shoots to be produced, which typically fail to elongate; they may also cause the leaves of some species to have an unusual shape, and/or induce shoots to become hyperhydric.

7.4.2. Adventitious shoot bud formation

The formation of adventitious shoots, whether directly from explanted tissues, or indirectly from callus, is regulated by an interaction between auxins and cytokinins.

7.4.3. Inhibition of root formation

High concentrations of cytokinin (0.5-10 mg/l) generally inhibit or delay root formation (Schraudolf and Reinert, 1959; Harris and Hart, 1964; Ben-Jaacov *et al*., 1991) and also prevent root growth and the promotive effects of auxins on root initiation

(Humphries, 1960). For this reason cytokinins are usually omitted from shoot culture media at Stage III when shoots are to be rooted to provide plantlets (see Chapter 2). Sometimes more than one subculture to a cytokinin-free medium may be required until the level of cytokinin within the tissues has been sufficiently reduced.

Despite these observations, there are reports that cytokinins can sometimes induce or promote root growth (Fries, 1960), or adventitious root formation, in the absence of auxins (Nemeth, 1979). In nearly all cases only low rates of cytokinin have been effective, for example, shoots of sugar beet were rooted on MS medium containing 0.5 mg/l kinetin and no auxin (Konwar and Coutts, 1990). Boxus and Terzi (1988) advocated the addition of 0.5 mg/l kinetin and auxin to the rooting medium for strawberries and several woody plants, finding that at this concentration, the cytokinin had a bacteriostatic effect and rooting was not impaired. *Rosa hybrida* 'White Dream' required the addition of 1 mg/l BA to IBA for root induction and development.

7.5. SPECIFICITY OF ACTION

The effect of cytokinins on tissue or organ cultures can vary according to the particular compound used, the type of culture, the variety of plant from which it was derived and whether the explant is derived from juvenile or mature tissues. In *Corylus avellana*, 5 mg/l BA gave the best rate of shoot multiplication from juvenile explants, but 10 mg/l zeatin was required for nodal sections of plants in the adult phase (Messeguer and Mel, 1987).

A requirement for a particular cytokinin is sometimes noted for the induction of embryogenesis (e.g. Fujimura and Komamine, 1975), and for the promotion of direct or indirect adventitious shoot formation; for example, cultures of *Browallia viscosa* required iP for the initiation of adventitious shoots. Kinetin, BA and zeatin were ineffective (Welsh and Sink, 1981).

7.6. CYTOKININ SPECIFICITY IN SHOOT CULTURES

Most demonstrations of a requirement for a particular cytokinin, have been made with shoot cultures; they are dispersed over many species. BA promoted axillary bud proliferation of *Castanea* in the experiments of Vieitez and Vieitez (1980b), whereas kinetin was without effect. Zeatin tended to promote the growth of main shoots and gave only a slight increase in the proportion of lateral buds sprouting. Similarly iP and kinetin produced only single shoots from *Prunus* shoot cultures: to obtain multiple shoots, it was necessary to use BA (Martinelli, 1985).

Elliott (1970) found kinetin to be incapable of promoting the growth of rose shoot tips. On the other hand, only 0.5-5 mg/l kinetin (together with gibberellic acid) induced the proliferation of potato shoots, and BA and iP were not effective. In some cultures, cytokinins tend to produce short rosetted shoots, or shoots which grow only slowly after formation: BA and kinetin gave shoot rosettes in *Brassica campestris* shoot cultures, whereas axillary shoots induced by iP elongated satisfactorily (Paek *et al.*, 1987): iP and BA produced many shoots of *Elaeagnus angustifolia*, but unlike those produced on 5 μM kinetin, they failed to grow afterwards (Bertrand and Lalonde, 1985). BA gives a high rate of shoot proliferation in *Gerbera*, but the best shoot quality is obtained using 5-10 mg/l kinetin (Pierik *et al.*, 1982; Hempel *et al.*, 1985).

Fonnesbech *et al.* (1979) discovered that the natural cytokinins iP and zeatin were better able to promote the growth and survival of shoot cultures of *Asparagus plumosus* than kinetin or BA, although best results were obtained with PBA. A similar situation is found in plants of the family Ericaceae, where the natural compounds, zeatin and iP, are more effective than other cytokinins for shoot proliferation. iP is most commonly selected because of its lower cost, but in some species, mixtures of the two compounds may give better results than either compound alone (Eccher and Noe, 1989).

In shoot cultures of *Gynura sarmentosa*, BA, kinetin and iP promoted the formation of buds when used separately, but each in turn produced some abnormality in the shoots obtained. Much faster growth of healthy shoots was obtained by adding all three compounds simultaneously (Cailloux, 1978). A mixture of more than one cytokinin has also been found to give more effective shoot multiplication in some other species (e.g. *Corylus avellana*, Anderson, 1984; *Cucumis melo*, Kathal *et al.*, 1988). Plant abnormalities associated with cytokinin use are mentioned in Chapter 13.

7.7. PHENYLUREAS

In some tests, phenylureas are much more effective cytokinins than the adenine-based compounds. 4PU-Cl was, for example, 100 times more active than BA in the tobacco callus assay (Read *et al.*, 1986) and produced more shoots in azalea shoot cultures than zeatin or iP. In many azalea (*Rhododendron*) cultivars, thidiazuron

produced many shoots but these were of poor quality because they were stunted and hyperhydric. Better results were obtained with a mixture of iP and thidiazuron, but there was a tendency for adventitious shoots to be produced, which is not desirable during micropropagation (Briggs *et al.*, 1988). Thidiazuron was adopted for the micropropagation of woody plants in the family Oleaceae (Einset and Alexander, 1985).

7.8. THE EFFECT OF TEMPERATURE

Maintaining *in vitro* cultures at abnormally high temperatures is reported to reduce the efficacy of cytokinins, but may enhance auxin activity. Heide (1965) observed that the ability of cytokinin-dips to either promote direct bud formation or inhibit direct root formation, was less when *Begonia* leaf cuttings were kept at 27°C after treatment, rather than at 15°C. On a modified MS medium containing kinetin, growth and axillary shoot proliferation of *Asparagus plumosus* shoot tips was initially rapid at 24°C but stopped after four weeks. The shoot tips grew more slowly at 17°C but their growth was continuous. There was almost no growth at all at either 9°C or 30°C (Fonnesbech *et al.*, 1977). The most effective cytokinin for A. *plumosus* was subsequently found to be PBA. The requirement for this cytokinin increased with temperature. At 17°C , the optimum dose was 0.2 mg/l, at 21°C the optimum was between 0.2 and 2 mg/l, but at 24°C it was 2 mg/l. The best temperature for survival, growth and development was 21°C (Fonnesbech *et al.*, 1979).

8. ADENINE

A possible growth regulatory effect caused by adenine (18) was first noted by Bonner and Haagen-Smit (1939) and Bonner *et al.* (1939), who found that the compound promoted an expansion in the area of leaf discs floated on sugar solutions. Other regulatory properties of the compound were later demonstrated by Skoog and Tsui (1948), Jacquiot (1951) and Miller and Skoog (1953), who discovered that it could induce bud formation in both tobacco stem segments and elm and tobacco callus *in vitro.* The activity of adenine is much less than that of the true cytokinins, and 25-100 times the concentration (e.g. 600 mM vs. 20 mM kinetin - Khanna and Chopra, 1977) may be required to produce similar results.

Despite the subsequent discovery of cytokinin activity in adenine derivatives, adenine itself is still often used in cultures from which plant regeneration is required. It seems sometimes to improve growth (Nwankwo and Krikorian, 1983), or to bring about or reinforce responses normally attributed to cytokinin action. It is not active in the soybean callus bioassay. Benefits are often only noticed when adenine is administered together with a cytokinin such as kinetin, or BA. Adenosine and adenylic acid can sometimes act in the same way as adenine (Skoog and Tsui, 1948; Nitsch *et al.*, 1967) but they are generally even less effective. Adenine is known as Vitamin B4. In some papers on tissue culture it is listed amongst the vitamin components of a medium.

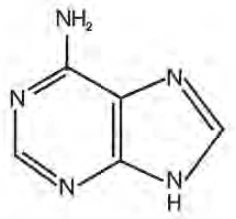
8.1. EMBRYOGENESIS AND CAULOGENESIS

Halperin and Wetherell (1964) noted that 2 mg/l adenine or 0.2 mg/l kinetin could be used instead of coconut milk in various media for stimulating embryogenesis in carrot callus. Since then, adenine has been added to media in amounts ranging from 2 to 405 mg/l (but more usually 40-80 mg/l) to promote somatic embryo formation in other callus cultures (Nag and Johri, 1969; Danilina, 1972; Pareek and Chandra, 1978b; Phillips and Collins, 1980; Reynolds *et al.*, 1980). In the presence of other recognised cytokinins, adenine frequently promotes adventitious shoot formation, indirectly from callus (Plummer and Leopold, 1957; Earle and Torrey, 1965; Thorpe and Murashige, 1968; Beach and Smith, 1979; Xiang-can *et al.*, 1989), or directly from explants (Ziv *et al.*, 1970; Start and Cumming, 1976; Seabrook *et al.*, 1976; Nickerson, 1978; Rao and Bapat, 1978). It inhibits root initiation (Doerschug and Miller, 1967) but has been reported to stimulate the growth of preformed roots of *Citrus* embryoids (Kochba *et al.*, 1974) and lupin seedlings (Fries, 1960) in a similar fashion to low levels of cytokinin.

8.2. SHOOT CULTURES

Adenine has been used only to a limited extent in meristem or shoot cultures, but has been employed occasionally to enhance the growth of isolated meristem tips (Kassanis, 1957; Elliott, 1970) and as an aid (or occasionally as an essential component) to induce the proliferation of axillary shoots in shoot

cultures (Anderson, 1975; Hennen and Sheehan, 1978; Harris and Stevenson, 1979; Pyott and Converse, 1981; Huang, 1984). Whether improved shoot multiplication will result from adding adenine to the medium can be unpredictable. In peach shoot cultures, a benefit depended on the cultivar being cultured and the nature and rate of the cytokinin used. A stimulation occurred with some rates of BA, but with others its addition resulted in a decreased rate of propagation (Chiariotti and Antonelli, 1988).



(18) adenine

Although the rate of *Brassica campestris* shoot multiplication was not increased, adding 326-434 μM adenine sulphate to a medium containing kinetin and IBA, caused shoot weight to be increased. Leaves were dark green and the cultures more healthy than previously (Paek *et al.*, 1987).

8.3. MODE OF ACTION

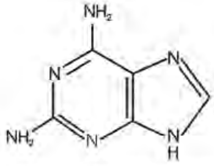
The mode of action of adenine has not been fully explained. Beneficial effects from adenine addition are found in media containing both ammonium nitrate and cytokinins (Ziv *et al.*, 1970; Elliott, 1970; Seabrook *et al.*, 1976; Nickerson, 1978; Pyott and Converse, 1981). Adenine is therefore unlikely to be simply acting as an alternative source of reduced nitrogen, and if it enhances natural cytokinin biosynthesis, the compounds produced must be more effective in causing the required physiological response than cytokinins added to the growth medium. Such a situation was described by Beach and Smith (1979) with callus of red and crimson clover. On Gamborg *et al.* (1968) B5 medium, plant regeneration was most effectively achieved when 20 mg/l thiamine, 2.0 mg/l NAA and 2 mg/l adenine were included. Kinetin, iP and BA were all tested as cytokinins and failed to produce shoot buds. However, in many cultures, adenine acts more as a synergist of cytokinins such as kinetin and zeatin (Nitsch *et al.*, 1967). A typical result is that of Start and Cumming (1976), who found that when 125 mg/l adenine was added to a medium in conjunction with 5 mg/l BA and 0.1 mg/l NAA, it regularly increased direct shoot initiation from African violet leaf sections, whereas in the basal medium with only adenine sulphate, shoot formation was delayed and roots alone were formed.

Both natural and synthetic purine-based cytokinins are degraded in plant tissues to adenine and related nucleotides (Terrine *et al.*, 1972; Entsch *et al.*, 1980; Biondi *et al.*, 1984; McGaw *et al.*, 1984; Forsyth and Van Staden, 1987). Thus one possible method whereby adenine may enhance the growth of cultured plants, could be that an excess of the compound retards the degradation of cytokinins by feed-back inhibition, or by competing for the enzyme systems involved in cytokinin metabolism. Another hypothesis is that, even though adenine is itself a product of cytokinin metabolism, it may act as a substrate for the synthesis of natural cytokinin growth substances. It has been shown that adenine can serve as a precursor for zeatin synthesis, but the rate of incorporation is low (McGaw *et al.*, 1984; Dickinson *et al.*, 1986).

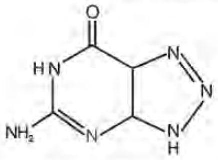
8.4. INHIBITORY EFFECTS

The addition of adenine does not always lead to beneficial results; for instance, adenine sulphate in the concentration range 30-300 mg/l was inhibitory for the formation of shoots from cultured tuber discs of potato (Jarret *et al.*, 1980). The addition of adenine sulphate to shoot cultures of carnation caused axillary shoot formation to be inconsistent; as a smaller number of shoots was produced from each shoot tip, and the main shoots often grew well, Davis *et al.* (1977) thought that the compound tended to enhance apical dominance. Where adenine is promotory, an excess can have the same consequences as an excess of cytokinin; Nickerson (1978) found that 80 mg/l adenine, enhanced adventitious bud formation in cotyledons and hypocotyl sections of lowbush blueberry, but needed to be withdrawn to promote shoot elongation.

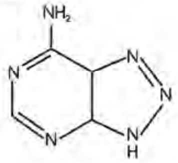
9. CYTOKININ ANTAGONISTS



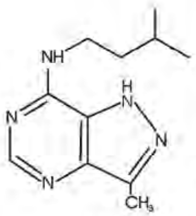
(19) 2,6-diaminopurine



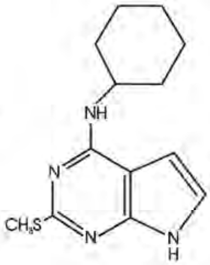
(20) 8-azaguanine



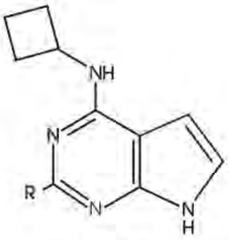
(21) 8-azaadenine



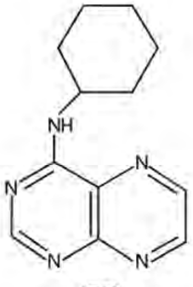
(22)



(23)

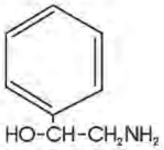


(24) R = CH_3
4-(cyclobutylamino)-2-methylpyrrolo[2,3-*d*]pyrimidine
(25) R = CH_3S
4-(cyclobutylamino)-2-methylthiopyrrolo[2,3-*d*]pyrimidine

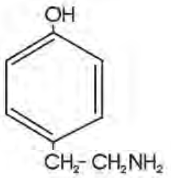


(26)
4-cyclopentylaminopteridine

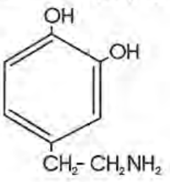
Certain chemical analogues of RNA bases can antagonise the action of cytokinins. Blaydes (1966) found that 2,6-diaminopurine (19), 8-azaguanine (20) and 8-azaadenine (21) inhibited the growth of kinetin-requiring soybean callus. Compounds which reversibly inhibit the growth of cytokinin-dependent and cytokinin-requiring callus cultures were described by Hecht *et al.* (1971), Skoog *et al.* (1973, 1975) and Gregorini and Laloue (1980). These authors found that the growth inhibitory effects of compounds such as 7-(3-methylbutylamino)-3-methyl-H-pyrazolo[4,3-d]-pyrimidine (22) and 4-cyclohexylamino)-2-methylthio-H-pyrrolo[2,3d]pyrimidine (23) were reversed by BA. Cell death by cytotoxicity was expressed only when the tobacco cells divided. Non-dividing cells in stationary phase were insensitive to either compound which suggests that a specific biochemical event in the cell cycle was affected. Cytokinin-autonomous callus is rendered cytokinin-requiring by these chemicals.



(27) octopamine



(28) tyramine



(29) dopamine

Other highly active antagonists of cytokinin-promoted cell division are 4-(cyclobutylamino)-2-methylpyrrolo [2,3-d] pyrimidine (24) (Iwamura *et al.*, 1979) and its 2-methylthio analogue (25) (Hecht, 1980), and 4-cyclopentylaminopteridine (26) (Iwamura *et al.*, 1980). Although the compounds mentioned above inhibit cytokinin-promoted cell division, they have not, in all cases, been shown to antagonise other physiological processes normally promoted by cytokinin (such as the stimulation of lateral bud growth, or the initiation of shoot buds in callus tissues). However, Tanimoto and Harada (1982) used a cytokinin antagonist (4-cyclopentylamino-2-methylpyrrolo[2,3-d]pyrimidine), to overcome the stimulation of adventitious bud formation in *Torenia* stem segments by both BA and the phenylurea, 2Cl-4PU. Some compounds which act as anticytokinins in preventing growth of tobacco callus, have been found to promote bud formation in

conjunction with an auxin (Skoog *et al*., 1975; Iwamura *et al*., 1979). This has led to speculation that there may be separate receptor sites for individual cytokinin-dependent functions (Hecht, 1980).

Anticytokinin activity also exists in compounds structurally related to octopamine (27), such as tyramine (28) and dopamine (29) (Christou and Barton, 1989).

10. AUXIN-CYTOKININ INTERACTION

Skoog and Miller (1957) found that shoot formation could be induced predictably from tobacco callus using relatively low levels of auxin and a high level of cytokinin in the growth medium. Since this discovery, many aspects of cellular differentiation and organogenesis in tissue and organ cultures have been found to be controlled by an interaction between cytokinin and auxin concentrations. The balance between the two sorts of regulant that is usually required to initiate growth or differentiation in tissue cultures, is illustrated in Fig. 6.3.

Relative proportions of auxins and cytokinins do not always produce the typical results shown in the figure. For example:

- axillary shoot proliferation in some species may be promoted by the presence of an auxin together with cytokinin;
- tissues from monocotyledons can often be induced to form callus by culture in high levels of auxin alone, and cytokinins may be non-essential or unimportant;
- organogenesis in monocotyledons is often promoted by transferring the culture to a medium without auxin, by reducing the concentrations of a highly active auxin such as 2,4-D, or replacing 2,4-D with another auxin (e.g. IAA or NAA).

A balance between auxin and cytokinin growth regulators is most often required for the formation of adventitious shoot and root meristems. The requisite concentration of each type of regulant differs greatly according to the kind of plant being cultured, the cultural conditions and the compounds used; interactions between the two classes of regulant are often complex, and more than one combination of substances is likely to produce optimum results.

10.1. CONTOUR DIAGRAMS

Results of growth regulator interactions can be difficult to express graphically without resorting to three dimensional graphs. Two dimensional alternatives are island diagrams (Negrutiu *et al.*, 1978), or contour diagrams (Frett and Smagula, 1983). The latter are a particularly effective means of presentation and can be used to summarize the results of experiments in which several different concentrations of an auxin and a cytokinin (or other combinations of regulants) have been combined. The position of contour lines can be estimated from results, but are most accurately plotted using equations derived from multiple regression. For such statistical analysis to be possible, a properly designed experiment must have been conducted (Beretta and Eccher, 1987). Fig. 6.4, drawn from results of Saunders and Bingham (1975), shows how combinations of kinetin and 2,4-D (in conjunction with a constant rate of NAA), influenced the percentage of *Medicago sativa* calluses producing adventitious shoots. A further example of a contour diagram is given in Chapter 7.

10.2. PRETREATMENTS

Similar effects to those produced by having auxin and cytokinin together in the medium, can often be produced by pretreatment with one regulant, followed by transfer to a second medium containing another. For example, preculturing peach leaf pieces on a medium containing 2,4-D, increased the callus production obtained when explants were placed on a medium with NAA and BA. If preculture with 2,4-D was followed by subculture to a medium with BA alone, callus was produced which gave rise to roots (Hammerschlag, 1988).

Despite the frequent need for both an auxin and a cytokinin in tissues cultures, the nature of the interactions between the two types of regulant is seldom commented upon and there is much still to be learned. Although both auxin and cytokinin are usually required for growth or morphogenesis, auxin can inhibit cytokinin accumulation (Hansen *et al.*, 1985) while cytokinins can inhibit at least some of the action of auxin (see above).

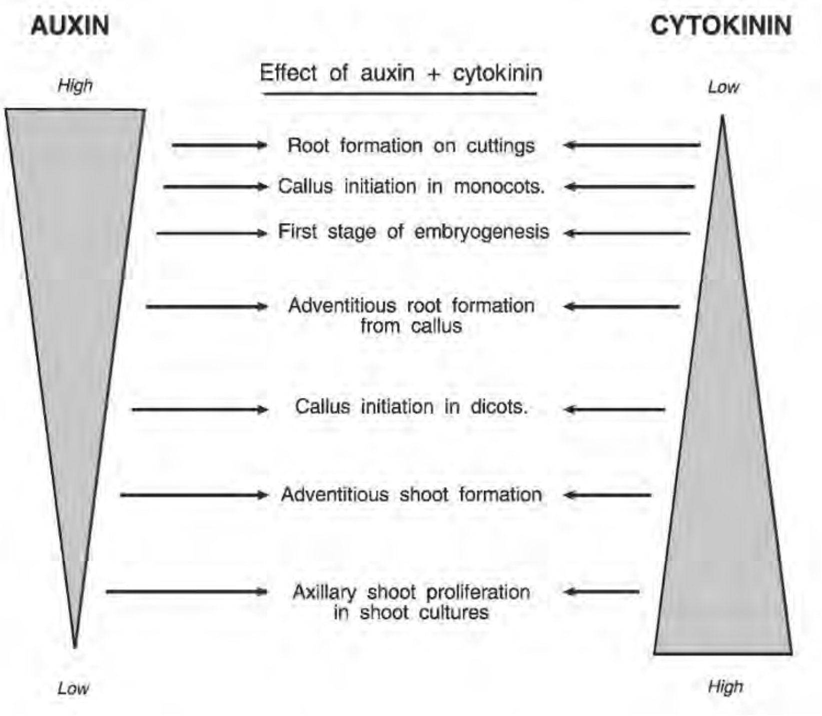


Fig. 6.3 The relative concentrations of auxin and cytokinin typically required for growth and morphogenesis.

10.3 GROWTH REGULATORS AND THE CELL CYCLE

Cell division seems to be regulated by the joint action of auxins and cytokinins, each of which appears to influence different phases of the cell cycle. Auxins exert an effect on DNA replication, while cytokinin seem to exert some control over the events leading to mitosis (Jouanneau, 1971; John *et al.*, 1993; Pasternak *et al.*, 2000, cf. Mode of action, above). Therefore auxins might be considered as 'inducers' of the cell cycle while cytokinins might behave more as its 'promoters' (Wood *et al.,* 1990). Normal cell divisions require synchrony between the S phase and cell division, suggesting that auxin and cytokinin levels in cultures need to be carefully matched. Late replication of DNA in cell cultures has been advanced as one cause of chromosome rearrangement (Lee and Phillips, 1988). Cells are thought not to enter mitosis unless cytokinin is present. Where callus or suspension cultures are initiated on media which only contain an auxin, reliance is presumably being placed on endogenous cytokinins for completion of the cell cycle?

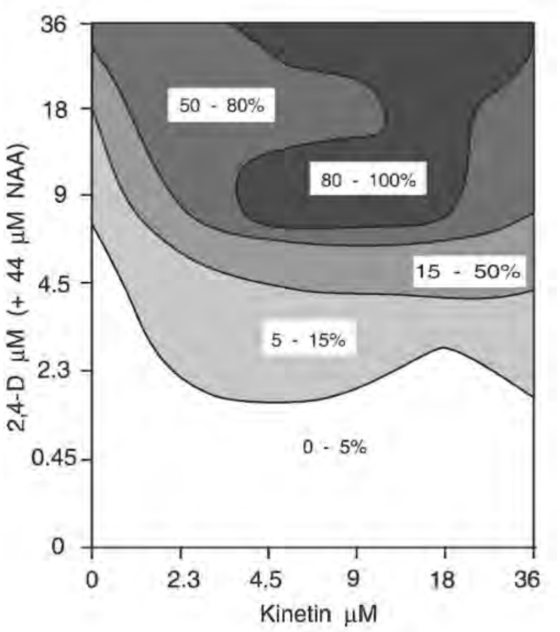


Fig. 6.4 A contour diagram drawn from the results of Saunders and Bingham (1975). The diagram shows the percentage of alfalfa calluses producing adventitious shoots when incubated on media containing various combinations of auxin and cytokinin.

REFERENCES

AKIYOSHI D.E., MORRIS R.O., HINZ R., MISCHKE B.S., KOSUGE T., GARFINKEL D.J., GORDON M.P. & NESTER E.W. 1983 Cytokinin/auxin balance in crown gall tumors is regulated by specific loci in the T-DNA. Proc. Natl. Acad. Sci. USA 80, 407-411.

ANDERSON W.C. 1984 Micropropagation of filberts *Coryllus avellana*. Comb. Proc. Int. Plant Prop. Soc. 1983, 33 132-137.

ANDERSON W.C. 1975 Propagation of rhododendrons by tissue culture. I. Development of a culture medium for multiplication of shoots. Comb. Proc. Int. Plant Prop. Soc. 25, 129-135.

ARNDT F., RUSCH R. & STILLFRIED H.V. 1976 SN49537, a new cotton defoliant. Plant Physiol. 57, 599.

ÅSTOT C., DOLEZAL K., NORDSTRÖM A., WANG Q., KUNKEL T., MORITZ T., CHUA N-H. & SANDBERG G. 2000 An alternative cytokinin biosynthesis pathway. Proc. Natl. Acad. Sci. USA 97, 14778-14783.

BARALDI R., ROSSI F. & LERCARI B. 1988 *In vitro* shoot development of *Prunus* GF655-2: interaction between light and benzyladenine. Physiol. Plant. 74, 440-443.

BASSIL N.V., MOK D.W.S. & MOK M.C. 1993 Partial purification of a *cis-trans*-isomerase of zeatin from immature seed of *Phaseolus vulgaris* L. Plant Physiol. 102, 867-872.

BEACH K.H. & SMITH R.R. 1979 Plant regeneration from callus of red and crimson clover. Plant Sci. Lett. 16, 231-237.

BEN-JAACOV J., ACKERMAN A., TAL E. & JACOBS G. 1991 Vegetative propagation of *Alberta magna* by tissue culture and grafting. HortScience 26, 74.

BERETTA D. & ECCHER T. 1987 Determination of optimum level of BAP, GA3 and IAA in micropropagation of *Camellia* by an orthogonal composite design. Acta Hortic. 212, 151-154.

BERTRAND L.J. & LALONDE M. 1985 *In vitro*-propagation and nodulation by *Frankia* of actinorhyzal Russian olive (*Elaeagnus angustifolia* L.). Plant and Soil 87, 143-152.

BINAROVA P., DOLEZEL J., DRABER P., HEBERLE-BORS E., STRNAD M. & BOGRE L. 1998 Treatment of *Vicia faba* root tip cells with specific inhibitors to cyclin-dependent kinases leads to abnormal spindle formation. Plant J. 16, 697-707.

BIONDI S., CANCIANI L. & BAGNI N. 1984 Uptake and translocation of benzyladenine by elm shoots cultured *in vitro*. Can. J. Bot. 62, 2385-2390.

BLAKESLEY D. & LENTON J.R. 1987 Cytokinin uptake and metabolism in relation to shoot multiplication *in vitro*. pp. 87-99 in Jackson *et al.* (eds.) 1987a (q.v.).

BLAYDES D.F. 1966 Interaction of kinetin and various inhibitors in the growth of soybean tissue. Physiol. Plant. 19, 748-753.

BOLLMARK M., KUBAT B. & ELIASSON L. 1988 Variation in endogenous cytokinin content during adventitious root formation in pea cuttings. J. Plant Physiol. 132, 262-265.

BONNER D.M. & HAAGEN-SMIT A.J. 1939 Leaf growth factors II. The activity of pure substances in leaf growth. Proc. Nat. Acad. Sci. U.S.A. 25, 184-188.

BONNER D.M., HAAGEN-SMIT A.J. & WENT F.W. 1939 Leaf growth hormones. 1. A bio-assay and source for leaf growth factors. Bot. Gaz. 101, 128-144.

BOXUS Ph. & TERZI J.M. 1988 Control of accidental contaminations during mass propagation. Acta Hortic. 225, 189-190.

BRIGGS B.A., McCULLOCH S.M. & EDICK L.A. 1988 Micropropagation of azaleas using thiadiazuron. Acta Hortic. 226, 205-208.

BRUCE M.I. & ZWAR J.A. 1966 Cytokinin activity of some ureas and thioureas. Proc. Roy. Soc. (Lond.) Ser. B 165, 245-265.

BRUCE M.I., ZWAR J.A. & KEFFORD N.P. 1965 Chemical structure and plant kinin activity; the activity of urea and thiourea derivatives. Life Sci. 4, 461-466.

BURROWS W.J., SKOOG F. & LEONARD N.J. 1971 Isolation and identification of cytokinins located in the transfer ribonucleic acid of tobacco callus grown in the presence of 6-benzylaminopurine. Biochemistry 10, 2189-2194.

BUTENKO R.G., ATANASSOV A. & URMANTSEVA W. 1972 Some features of the sugar beet tissue culture. Phytomorph. 22, 140-143.

CAILLOUX M. 1978 Simultaneous use of 3 cytokinins gives better yields with *Gynura sarmentosa*. p. 248 in Hughes *et al.* (eds.) 1978 (q.v.).

CARMAN J.G. & CAMPBELL W.A. 1988 Factors affecting somatic embryogenesis in wheat. In Bajaj Y.P.S. (ed.) 1988. *Biotechnology in Agriculture and Forestry. Crops II.* Springer-Verlag, Berlin, Heidelberg, New York.

CARR D.J. (ed.) 1972 *Plant Growth Substances* 7th Int. Conf. on Plant Growth Subs. 1970 Springer- Verlag, Berlin. Heidelberg, New York.

CHATFIELD J.M. & ARMSTRONG D.J. 1988 Cytokinin oxidase from *Phaseolus vulgaris* callus cultures. Affinity for concanavalin A. Plant Physiol. 88, 245-247.

CHEN C.-M., ERTL J.R., LEISNER S.M. & CHANG C.-C. 1985 Localization of cytokinin biosynthetic sites in pea plant and carrot roots. Plant Physiol. 78, 510-513.

CHIARIOTTI A. & ANTONELLI M. 1988 The effect of 6-BAP and adenine sulphate on peach shoot proliferation. Acta Hortic. 227, 418-420.

CHRISTOU P. & BARTON K.A. 1989 Cytokinin antagonist activity of substituted phenethylamines in plant cell culture. Plant Physiol. 89, 564-568.

CROZIER A. & HILLMAN J.R. (eds.) 1984 *The Biosynthesis and Metabolism of Plant Hormones.* Soc. for Exp. Biology, Seminar Series 23, Cambridge Univ. Press ISBN 0-521-26424-3.

DANILINA A.N. 1972 Morphogenesis of embryoids from carrot tissue cultures. Phytomorph. 22, 160-164.

DAVIS M.J., BAKER R. & HANAN J.J. 1977 Clonal multiplication of carnation by micropropagation. J. Am. Soc. Hortic. Sci. 102, 48-53.

DE AZEVEDO W.F., LECLERC S., MEIJER L., HAVLICEK L., STRNAD M. & KIM S.-H. 1997 Inhibition of cyclin-dependent kinases by purine analogues. Crystal structure of human cdk2 complexed with roscovitine. Eur. J. Biochem. 243, 518-526.

DEN BOER B.G.W. & MURRAY J.A.H. 2000 Triggering the cell cycle in plants. Trends Cell Biol. 10, 245-250.

DERUERE J & KIEBER J.J. 2002 Molecular mechanisms of cytokinin signalling. J. Plant Growth Regul. 21, 32-39.

DICKINSON J.R., FORSYTH C. & VAN STADEN J. 1986 The role of adenine in the synthesis of cytokinins in tomato plants and in cell-free root extracts. Plant Growth Regul. 4, 325-334.

DOERSCHUG M.R. & MILLER C.O. 1967 Chemical control of adventitious organ formation in *Lactuca sativa* explants. Am. J. Bot. 54, 410-413.

DOMINOV J.A., STENZLER L., LEE S., SCHWARZ J.J., LEISNER S. & HOWELL S.H. 1992 Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. Plant Cell 4, 451-461.

DYSON W.H. & HALL R.H. 1972 N6-(D2-isopentenyl) adenosine: its occurrence as a free nucleoside in an autonomous strain of tobacco tissue. Plant Physiol. 50, 616-621.

EARLE E.D. & TORREY J.G. 1965 Morphogenesis in cell colonies grown from *Convolvulus* cell suspensions plated on synthetic media. Am. J. Bot. 52, 891-899.

ECCHER T. & NO N. 1989 Comparison between 2iP and zeatin in the micropropagation of highbush blueberry (*Vaccinium corymbosum*). Acta Hortic. 241, 185-190.

EINSET J.W. & ALEXANDER J.H. (III) 1985 Multiplication of *Syringa* species and cultivars in tissue culture. Comb. Proc. Int. Plant Prop. Soc. 1984 34, 628-636.

EINSET J.W. & SKOOG F. 1973 Biosynthesis of cytokinins in cytokinin-autotrophic tobacco callus. Proc. Nat. Acad. Sci. U.S.A. 70, 658-660.

EINSET J.W. 1986a Zeatin biosynthesis from N6-(D2-iso-pentenyl)adinine in *Actinidia* and other woody plants. Proc. Nat. Acad. Sci. U.S.A. 83, 972-975.

EINSET J.W. 1986b Role of cytokinin in shoot cultures of woody species. p. 298 in Somers *et al.* (eds.) 1986 (q.v.).

ELLIOTT R.F. 1970 Axenic culture of meristem tips of *Rosa multiflora*. Planta 95, 183-186.

ELOBEIDY A. & KORBAN S.S. 1988 The effect of thidiazuron on shoot regeneration from apple leaf discs. HortScience 23, 755.

ELSTNER E.F., KELLER G. & PARADIES I. 1983 Contrasting effects of the cotton defoliant thidiazuron and aminoethoxy-vinylglycine, an inhibitor of ethylene formation, on stomatal aperture and on ethylene formation in bean (*Phaseolous vulgaris*) leaves. Ber. Deutsch. Bot. Ges. 96,459-467.

EMERY R.J.N., LEPORT L., BARTON J.E., TURNER N.C. & ATKINS C.A. 1998 *cis*-Isomers of cytokinins predominate *Cicer arietinum* throughout their development. Plant Physiol. 117, 1515-1523.

ENTSCH B., LETHAM D.S., PARKER C.W., SUMMONS R.E. & GOLLNOW B.I. 1980 Metabolites of cytokinins. pp. 109-118 in Skoog (ed.) 1980 (q.v.).

ERNST D. & OESTERHELT D. 1985 Changes of cytokinin nucleotides in an anise cell culture (*Pimpinella anisum* L.) during growth and embryogenesis. Plant Cell Rep. 4, 140-143.

ERNST D., SCHÄFER W. & OESTERHOLT D. 1983 Isolation and identification of a new, naturally occurring cytokinin (6-benzyl-aminopurine riboside) from an anise cell culture (*Pimpinella anisum* L.). Planta 159, 222-225.

ESTÉVEZ J.M., REINDL A., REICHLER S. & LEÓN P. 2001 1-Deoxy-D-xylulose-5-phosphate, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. J. Biol. Chem. 276, 22901-22909.

FASOLO F., ZIMMERMAN R. H., FORDHAM I. & FALBO L. 1988a Adventitious shoot formation on excised leaves of *in vitro* cultured apples: effects of cytokinin, auxin, and leaf age. HortScience 23, 755.

FASOLO F., ZIMMERMAN R.H. & FORDHAM I. 1988b Adventitious shoot formation on excised leaves *in vitro* cultured apple cultivars. HortScience 23, 676.

FELLMAN C.D., READ P.E. & HOSIER M.A. 1987 Effects of thidiazuron and CPPU on meristem formation and shoot proliferation. HortScience 22, 1197-1200.

FONNESBECH A., FONNESBECH M. & BREDMOSE N. 1977 Growth and development of *Asparagus plumosus* shoot tips *in vitro*. Acta Hortic. 78, 287.

FONNESBECH A., FONNESBECH M. & BREDMOSE N. 1979 Influence of cytokinins and temperature on development of *Asparagus plumosus* shoot tips *in vitro*. Physiol. Plant. 45, 73-76.

FORSYTH C. & VAN STADEN J. 1987 Cytokinin metabolism in tomato plants. II. Metabolites of kinetin and benzyladenine in decapitated roots. Plant Growth Regul. 6, 277-292.

FOX J.E. 1992 Molecular modeling of cytokinins and the CBF-1 receptor. In: *Physiology and Biochemistry of Cytokinins in Plants* (Kamínek M., Mok D.W.S., Zazimalová E. (eds.) pp. 127-132. SPB Academic Publishing, The Hague, The Netherlands.

FUJIWARA A. (ed.) 1982 *Plant Tissue Culture* 1982. Proc. 5th. Int. Cong. Plant Tiss. Cell Cult., Japan. Assoc. Plant Tissue Culture, Japan.

FRANK M. & SCHMÜLLING T. 1999 Cytokinin cycles cells. Trends Plant Sci. 4, 243-244.

FRETT J.J. & SMAGULA J.M. 1983 *In vitro* shoot production of lowbush blueberry. Can. J. Plant Sci. 63, 467-472.

FRIES N. 1960 The effect of adenine and kinetin on growth and differentiation of *Lupinus*. Physiol. Plant. 13, 468-481.

FUERST R.A.U.A., SONI R., MURRAY J.A.H. & LINDSEY K. 1996 Modulation of cyclin transcript levels in cultured cells of *Arabidopsis thaliana*. Plant Physiol. 112, 1023-1033.

FUJIMURA T. & KOMAMINE A. 1975 Effects of various growth regulators on the embryogenesis in a carrot cell suspension culture. Plant Sci. Lett. 5, 359-364.

GAMBORG O.L., MILLER R.A. & OJIMA K. 1968 Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151-158.

GILBY A.C. & WAINWRIGHT H. 1989 Use of tissue culture in improvement of watercress (*Rorippa nasturtium aquaticum* L. Hayek). Acta Hortic. 244, 105-113.

GREEN C.E, SOMMERS D.A., HACKETT W.P. & BIESBOER D.D. (eds.) 1987 *Plant Tissue and Cell Culture*. Alan R. Liss, Inc., New York.

GREGORINI G. & LALOUE M. 1980 Biological effects of cytokinin antagonists 7-(pentylamino) and 7-(benzylamino)-3-methylpyrazolo (4,3-d) pyrimidines on suspension-cultured tobacco cells. Plant Physiol. 65, 363-367.

GUPTA S.D. & HADLEY G. 1977 Phytotoxicity of benomyl on orchid seedlings. Am. Orchid Soc. Bull. 46, 905-907.

HABERER G. & KIEBER J.J. 2002 Cytokinins. New insights into a classic phytohormone. Plant Physiol. 128, 354-362.

HALPERIN W. & WETHERELL D.F. 1964 Adventive embryony in tissue cultures of the wild carrot, *Daucus carota*. Am. J. Bot. 51, 274-283.

HAMMERSCHLAG F.A. 1988 Callus and root formation from leaves of mature peach plants propagated *in vitro*. HortScience 23, 756.

HANSEN C.E., MEINS F. Jr. & AEBI R. 1987 Hormonal regulation of zeatin-riboside accumulation by cultured tobacco cells. Planta 172, 520-525.

HANSEN C.E., MEINS F. Jr. & MILANI A. 1985 Clonal and physiological variation in the cytokinin content of tobacco cell lines differing in cytokinin requirement and capacity for neoplastic growth. Differentiation 29, 1-6.

HARRIS G.P. & HART E.M.H. 1964 Regeneration from leaf squares of *Peperomia sandersii* A, DC: a relationship between rooting and budding. Ann. Bot. 28, 509-526.

HARRIS R.E. & STEVENSON J.H. 1979 Virus elimination and rapid propagation of grapes *in vitro*. Comb. Proc. Int. Plant Prop. Soc. 29, 95-108.

HAVLICEK L., HANUS J., VESELY J., LECLERC S., MEIJER L., SHAW G. & STRNAD M. 1997 Cytokinin-derived cyclin-dependent kinase inhibitors: Synthesis and cdc2 inhibitory activity of olomoucine and related compounds. J. Med. Chem. 40, 408-412.

HECHT S.M. 1980 Probing the cytokinin receptor site(s). pp. 144-160 in Skoog F. (ed.) 1980 (q.v.).

HECHT S.M., BOCK R.M., SCHMITZ R.Y., SKOOG F. & LEONARD N.J. 1971 Cytokinins: Development of a potent antagonist. Proc. Nat. Acad. Sci. U.S.A. 68, 2608-2610.

HEIDE O.M. 1965 Interaction of temperature, auxins and kinins in the regeneration ability of *Begonia* leaf cuttings. Physiol. Plant. 18, 891-920.

HEMPEL M., PETOS-WITKOWSKA B. & TYMOSZUK J. 1985 The influence of cytokinins on multiplication and subsequent rooting of *Gerbera in vitro*. Acta Hortic. 167, 301-305.

HENNEN G.R. & SHEEHAN T.J. 1978 *In vitro* propagation of *Platycerium stemaria*. HortScience 13, 245
HORGAN R. 1987a Plant growth regulators and the control of growth and differentiation in plant tissue cultures. pp. 135-149 in Green *et al.*(eds.) 1987 (q.v.).
HORGAN R. 1987b Cytokinin genes. pp. 119-130 in Hoad G.V., Lenton J.R., Jackson M.B. and Atkin R.K. (eds.) 1987 *Hormone Action in Plant Development – A Critical Appraisal*. Butterworths, London, Boston.
HORGAN R., HEWETT E.W., PURSE J. & WAREING P.F. 1973 A new cytokinin from *Populus robusta*. Tetrah. Lett. 30, 2827-2828.
HUANG L.-C. 1984 Alternative media and method for *Cattleya* propagation by tissue culture. Am. Orchid Soc. Bull. 53, 167-170.
HUGHES K.W., HENKE R. & CONSTANTIN M. (eds.) 1978 *Propagation of Higher Plants through Tissue Culture*. Technical Inf. Center, U.S. Dept. Energy. Spingfield, Va. CONF-7804111.
HUMPHRIES E.C. 1960 Kinetin inhibited root formation on leaf petioles of detached leaves of *Phaseolus vulgaris* (dwarf bean). Physiol. Plant. 13, 659-663.
HWANG I., SHEEN J. 2001 Two-component circuitry in *Arabidopsis* cytokinin signal transduction. Nature 413, 383-389
IMEL M.R. & PREECE J.E. 1988 Adventitious shoot forma-tion from recultured leaves of rhododendron. HortScience 23, 760.
INOUE T., HIGUCHI M., HASHIMOTO Y., SEKI M., KOBAYASHI M., KATO T., TABATA S., SHINOZAKI K. & KAKIMOTO T. 2001 Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. Nature 409, 1060-1063.
IWAMURA H., MASUDA N., KOSHIMIZU K. & MATSUBARA S. 1979 Cytokinin-agonist and antagonist activities of 4-substituted-2-methylpyrrolo 2.3-d pyrimidines, 7-deaza analogs of cytokinin-active adenine derivatives. Phytochemistry 18, 217-222.
IWAMURA H., MASUDA N., KOSHIMIZU K. & MATSUBARA S. 1980 Effects of 4-alkylaminopteridines on tobacco callus growth. Plant Sci. Lett. 20, 15-18.
JACKSON M.B., MANTELL S.H. & BLAKE J. (eds.) 1987a *Advances in the Chemical Manipulation of Plant Tissue Cultures*. Monograph 16, British Plant Growth Regulator Group, Bristol.
JACQUIOT C. 1951 Action of *meso*-inositol and of adenine on bud formation in the cambium tissue of *Ulmus campestris* cultivated *in vitro*. Compt. Rend. Acad. Sci. Paris 233, 815-817.
JARRET R.L., HASEGAWA P.M. & ERICKSON H.T. 1980 Effects of medium components on shoot formation from cultured tuber discs of potato. J. Am. Soc. Hortic. Sci. 105, 238-242.
JOHN P.C.L., ZHANG K., DONG CH., DIEDERICH L. & WIGHTMAN F. 1993 p34cdc2 related proteins in control of cell cycle progression, the switch between division and differentiation in tissue development, and stimulation of division by auxin and cytokinin. Aust. J. Plant Physiol. 20, 503-526.
JOUANNEAU J.-P. 1970 Renouvellement des protéines et effet spécifique de la kinétine sur des cultures de cellules de Tabac. Physiol. Plant. 23, 232-244.
JOUANNEAU J.P. 1971 Côntrole par les cytokinines de la synchronisation des mitoses dans les cellules de tabac. Exp. Cell Res. 67, 329-337.
JOUANNEAU J.P. 1975 Protein synthesis requirement for the cytokinin effect upon tobacco cell division. Exp. Cell Res. 91, 184-190
KAKIMOTO T. 1996 CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. Science 274, 982-985.
KAKIMOTO T. 2001. Identification of plant cytokinin bio-synthetic enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyl transferase. Plant Cell Physiol. 42, 677-685
KAMADA H. & HARADA H. 1979 Influence of several growth regulators and amino acids on *in vitro* organogenesis of *Torenia fournieri*. J. Exp. Bot. 30, 27-36.
KAMÍNEK M., VANÉK T., KALENDOVA-KULASOVA A. & PILAR J. 1987 The effect of two cytokinins on production of stem cuttings by stock plants of *Euphorbia pulcherrima* Willd. and *Gerbera jamesonii* Hook. Sci. Hortic. 33, 281-289.
KASSANIS B. 1957 The use of tissue cultures to produce virus-free clones from infected potato varieties. Ann. Appl. Biol. 45, 422-427
KATHAL R. BHATNAGAR S.P. & BHOJWANI S.S. 1988 Regeneration of plants from leaf explants of *Cucumis melo* cv. Pusa Sharbati. Plant Cell Rep. 7 449-451.
KEFFORD N.P., BRUCE M.I. & ZWAR J.A. 1966 Cytokinin activities of phenylurea derivatives - bud growth. Planta 68, 292-296.
KEMP T.R. & STOLTZ L.P. 1979 Cytokinins from growth regulator autonomous callus tissue. Plant Sci. Lett. 16, 403-408.
KERNS H.R. & MEYER M.M. Jr. 1986 Tissue culture propagation of *Acerx* x *freemanii* using thidiazuron to stimulate shoot tip proliferation. HortScience, 21, 1209-1210.
KHANNA R. & CHOPRA R.N. 1977 Regulation of shoot-bud and root formation from stem explants of *Lobularia maritima*. Phytomorph. 27, 267-274.
KIRKHAM M.B. & HOLDER P.L. 1981 Water osmotic and turgor potentials of kinetin-treated callus. HortScience 16, 306-307.
KOCHBA J., BUTTON J., SPIEGEL-ROY P., BORNMAN C.H. & KOCHBA M. 1974 Stimulation of rooting of citrus embryoids by gibberellic acid and adenine sulphate. Ann. Bot. 38, 795-802.
KODA Y. & OKAZAWA Y. 1980 Cytokinin production by asparagus shoot apex cultured *in vitro*. Physiol. Plant. 49, 193-197.
KONWAR B.K. & COUTTS R.H.A. 1990 Rapid regeneration of sugar beet (*Beta vulgaris* L.) plants from *in vitro* cultures. pp. 114-118 in Nijkamp *et al.* (eds.) 1990 (q.v.).
KULAEVA O.N. 1980 Cytokinin action on enzyme activities in plants. pp. 119-128 in Skoog F. (ed.) 1980 (q.v.).
KUROSAKI F., TAKAHASHI S., SHUDO K., OKAMOTO T. & ISOGAI Y. 1981 Structure and biological links between urea and purine cytokinins. Chem. Pharm. Bull. 29, 3751-3753.
LAUREYS F., DEWITTE W., WITTERS E., VAN MONTAGU M., INZÉ D. & VAN ONCKELEN H. 1998 Zeatin is indispensable for the G2-M transition in tobacco BY-2 cells. FEBS Lett. 426, 29-32.
LAUREYS F., SMETS R., LENJOU M., VAN BOCKSTAELE D., INZÉ D. & VAN ONCKELEN H. 1999 A low content of zeatin type cytokinins is not restrictive for the occurrence of G1/S transition in tobacco BY-2 cells. FEBS Lett. 460, 123-128.
LEE M. & PHILLIPS R.L. 1988 The chromosomal basis of somaclonal variation. Annu. Rev. Plant Physiol. 39, 413-417.
LEE T.T. 1974 Cytokinin control in subcellular localization of indoleacetic acid oxidase and peroxidase. Phytochemistry 13, 2445-2453.
LETHAM D.S. 1967 Chemistry and physiology of kinetin-like compounds. Annu. Rev. Plant Physiol. 18, 349-364.
LINSMAIER E.M. & SKOOG F. 1965 Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18, 100-127.
MÄHÖNEN A.P., BONKE M., KAUPINNEN L., RIIKONEN M., BENFEY P.N. & HELARIUTTA Y. 2000 A novel two-

component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root. Genes Dev. 14, 2938-2943.

MARIANO G. 1988 *In vitro* ^{14}C-labelled 6-benzyladenine uptake and CO_2 evolution in two Japanese plum cultivars. Plant Cell Tissue Organ Cult. 13, 49-59.

MARINO G. 1986 *In vitro* 6-benzylaminopurine purine uptake in two Japanese plums. Acta Hortic. 179, 871-872.

MARTINELLI A. 1985 Factors affecting *in vitro* propagation of the peach almond hybrids `Hansen 536'. Acta Hortic. 173, 237-244.

McGAW B.A., SCOTT I.M. & HORGAN R. 1984 Cytokinin biosynthesis and metabolism. pp. 105-133 in Crozier and Hillman (eds.) 1984 (q.v.).

MESSEGUER J. & MEL E. 1987 *In vitro* propagation of adult material and seedlings of *Corylus avellana*. Acta Hortic. 212, 499-503.

MILLER C. & SKOOG F. 1953 Chemical control of bud formation in tobacco stem segments. Am. J. Bot. 40, 768-773.

MILLER C.O. 1961a A kinetin-like compound in maize. Proc. Nat. Acad. Sci. U.S.A. 47, 170-174.

MILLER C.O. 1961b Kinetin related compounds in plant growth. Annu. Rev. Plant Physiol. 12, 395-408.

MILLER C.O. 1963 Kinetin and kinetin-like compounds. pp. 194-202 in Linskens & Tracey (eds.). *Modern Methods of Plant Analysis*. Vol. 6. Springer-Verlag, Berlin.

MILLER C.O. 1979 Cytokinin inhibition of respiration by cells and mitochondria of soybean. Planta 146, 503-511.

MILLER C.O. 1980 Cytokinin inhibition of respiration in mitochondria from six plant species. Proc. Nat. Acad. Sci. U.S.A. 77, 4731-4735.

MILLER C.O. 1982 Cytokinin modification of mitochondrial function. Plant Physiol. 69, 1274-1277.

MILLER C.O., SKOOG F., OKUMURA F.S., VON SALTZA M.H. & STRONG F.M. 1955b Structure and synthesis of kinetin. J. Am. Chem. Soc. 77, 2662-2663.

MILLER C.O., SKOOG F., VON SALTZA M. & STRONG F.M. 1955a Kinetin, a cell division factor from deoxyribonucleic acid. J. Am. Chem. Soc. 77, 1392.

MILLER M.W. & KUENERT C.C. *The Dynamics of Meristem Cell Populations*. Plenum Press, New York, London.

MOK M.C., MOK D.W.S., ARMSTRONG D.J., SHUDO K., ISOGAI Y. & OKAMOTO T. 1982 Cytokinin activity of N-phenyl-N-1,2,3-thiadiazol-5-ylurea (thiadiazuron). Phytochemistry 21, 1509-1511.

MOK, M.C., MARTIN, R.C., MOK, D.W.S. & SHAW, G. 1992 Cytokinin activity, metabolism and function in *Phaseolus*. pp. 41-46 in Kamínek M., Mok D.W.S., Zazímalová E. (eds.) 1992 *Physiology and Bio-chemistry of Cytokinins in Plants.* SPB Academic Publishing, The Hague, The Netherlands..

MOTYKA V. & KAMINEK M. 1990 Regulation of cytokinin catabolism in tobacco callus cultures. pp. 492-497 in Nijkamp *et al.* (eds.) 1990 (q.v.).

MOTYKA V., FAISS M., STRNAD M., KAMÍNEK M. & SCHMÜLLING T. 1996 Changes in cytokinin content and cytokinin oxidase activity in response to derepression of *ipt* gene transcription in transgenic tobacco calli and plants. Plant Physiol. 112, 1035-1043.

MUSGRAVE M.E. & SIEDOW J.N. 1985 A relationship between plant responses to cytokinins and cyanide resistant respiration. Physiol. Plant. 64, 161-166.

NAG K.K. & JOHRI B.M. 1969 Organogenesis and chromosomal constitution in embryo callus of *Nuytsia floribunda*. Phytomorph. 19, 405-408.

NEGRUTIU I., JACOBS M. & CACHITA D. 1978 Some factors controlling *in vitro* morphogenesis of *Arabidopsis thaliana*. Z. Pflanzenphysiol. 86, 113-124.

NEMETH G. 1979 Benzyladenine-stimulated rooting in fruit-tree rootstocks cultured *in vitro*. Z. Pflanzenphysiol. 95, 389-396.

NEUMANN K.H. 1968 Untersuchungen uber Beziehungen zwischen Zellteilung und Morphogenese bei Gewebekulturen von *Daucus carota*. II. Respiration von Gewebekulturen in Abhangigkeit von der Zellteilungsintensitat. Physiol. Plant. 21, 519-524.

NICKERSON N.L. 1978 *In vitro* shoot formation in lowbush Blueberry seedling explants. HortScience 13, 698.

NIJKAMP H.J.J., VAN DER PLAS I.H.W. & VAN AARTRIJK (eds.) 1990 *Progress in Plant Cellular and Molecular Biology*. Proc. VIIth Int. Cong. on Plant Tissue and Cell Culture. Amsterdam, The Netherlands. 24-29 June 1990. Kluwer Academic Publishers, Dortrecht, Netherlands.

NITSCH C. 1968 Induction *in vitro* de la floraison chez une plante de jours courts: *Plumbago indica* L. Ann. Sci. Nat. Bot. Paris, 12th Series 9, 1-91.

NITSCH J.P. & BUI DANG HA D. 1967 Effet de l'ordre d'application de l'acide indolyl-3-acetique et de la kinetine sur la division cellulaire. Compt. Rend. Acad. Sci. Paris 264D, 288-291.

NITSCH J.P., NITSCH C., ROSSINI L.M.E. & BUI DANG HA D. 1967 The role of adenine in bud differentiation. Phytomorph. 17, 446-453.

NWANKWO B.A. & KRIKORIAN A.D. 1983 Morpho-genetic potential of embryo- and seedling-derived callus of *Elaeis guineensis* Jacq. var. pisifera Becc. Ann. Bot. 51, 65-76.

OCHATT S.J. & POWER J.B. 1988 An alternative approach to plant regeneration from protoplasts of sour cherry (*Prunus cerasus* L.). Plant Sci. 56, 75-79.

OHYAMA K. & OKA S. 1982 Multiple shoot formation from Mulberry (*Morus alba* L.) hypocotyls by N-(2-chloro-4-pyridyl)-N-phenylurea. pp. 149-150 in Fujiwara A. (ed.) 1982 (q.v.).

OKAMOTO T., SHUDO K., TAKAHASHI S., YATSUNAMI T., ISOGAI Y. & YAMADA K. 1978 Shoot formation of tobacco callus by varous cytokinin-active ureas and pyrimidines. Chem. Pharm. Bull. 26, 3250-3252.

PAEK K.Y., CHANDLER S.F. & THORPE T.A. 1987 *In vitro* propagation of Chinese cabbage from seedling shoot tips. J. Am. Soc. Hort. Sci. 112, 841-845.

PALMER M.V. & PALNI L.M.S. 1987 Substrate effects on cytokinin metabolism in soybean callus tissue. J. Plant Physiol. 126, 365-371.

PAREEK L.K. & CHANDRA N. 1978b Differentiation of shoot buds *in vitro* in tissue cultures of *Sisymbrium irio*. J. Exp. Bot. 29, 239-244.

PASTERNAK T., MISKOLCZI P., AYAYDIN F., MÉSZÁROS T., DUDITS D. & FEHÉR A. 2000 Exogenous auxin and cytokinin dependent activation of CDKs and cell division in leaf protoplast-derived cells of alfalfa. Plant Growth Regul. 32, 129-141.

PATERSON K.E. & ROST T.L. 1981 Callus formation and organogenesis from cultured leaf segments of *Crassula argentea*: cytokinin-induced developmental pattern changes. Am. J. Bot. 68, 965-972.

PEAUD-LENOEL C. & JOUANNEAU J.-P. 1980 Presence and possible functions of cytokinins in RNA. pp. 129-143 in Skoog F. (ed.) 1980 (q.v.).

PHILLIPS G.C. & COLLINS G.B. 1980 Somatic embryogenesis from cell suspension cultures of red clover. Crop Sci. 20, 323-326.

PIERIK R.L.M., STEEGMANS H.H.M., VERHAEGH J.A.M. & WOUTERS A.N. 1982 Effect of cytokinin and cultivar on shoot formation of *Gerbera jamesonii in vitro*. Neth. J. Agric. Sci. 30, 341-346.

PLANCHAIS S., GLAB N., INZÉ D. & BERGOUNIOUX C. 2000 Chemical Inhibitors: a tool for plant cell cycle studies. FEBS Lett. 476, 78-83.

PLUMMER T.H. & LEOPOLD A.C. 1957 Chemical treatment for bud formation in *Saintpaulia*. Proc. Am. Soc. Hortic. Sci. 70, 442-444.

PYOTT J.L. & CONVERSE R.H. 1981 *In vitro* propagation of heat-treated red raspberry clones. HortScience 16, 308-309.

RAO P.S. & BAPAT V.A. 1978 Vegetative propagation of sandalwood plants through tissue culture. Can. J. Bot. 56, 1153-1156.

READ P.E., FELLMAN C.D. & ZIMMERMAN E. 1986 New plant growth regulators for cuttings and for tissue culture. Comb. Proc. Int. Plant Prop. Soc. 1985 35, 78-84.

REDIG P., SHAUL O., INZÉ D., VAN MONTAGU M. & VAN ONCKELEN H. 1996 Levels of endogenous cytokinins, Indole-3-acetic acid and abscisic acid during the cell cycle of synchronized tobacco BY-2 cells. FEBS Lett. 391, 175-180.

REISCH B.I. & MARTENS M.-H.R. 1988 Shoot regeneration from grape leaf, petiole and tendril tissues. HortScience 23, 807.

REYNOLDS B.D., BLACKMON W.J. & POSTEK C.E. 1980 Production of somatic embryos from Roselle callus. HortScience 15, 432 (Abst.447).

RODAWAY S. & LUTZ A.W. 1985 Nitroguanidines: a new class of synthetic cytokinins. Plant Physiol. 77, Suppl. 21 (Abst. 109).

ROGOZINSKA J.H., KROON C. & SALEMINK C.A. 1973 Influence of alterations in the purine ring on biological activity of cytokinins. Phytochemistry 12, 2087-2092.

ROTHWELL K. & WRIGHT S.T.C. 1967 Phytokinin activity in some new 6-substituted purines. Proc. Roy. Soc. 167B, 202-223.

SAUNDERS J.W. & BINGHAM E.T. 1975 Growth regulator effects on bud initiation in callus cultures of *Medicago sativa*. Am. J. Bot. 62, 850-855.

SCHMÜLLING T. 2002 New insights into the functions of cytokinins in plant development. J. Plant Growth Regul. 21, 32-39.

SCHRAUDOLF H. & REINERT J. 1959 Interaction of plant growth regulators in regeneration processes. Nature 184, 465-466.

SCOTT K.J., DALY J. & SMITH H.H. 1964 Effects of indoleacetic acid and kinetin on activities of enzymes of the hexose monophosphate shunt in tissue cultures of *Nicotiana*. Plant Physiol. 39, 709-712.

SEABROOK J.E.A., CUMMING B.G. & DIONNE L.A. 1976 The *in vitro* induction of adventitious shoot and root apices on *Narcissus* (daffodil and narcissus) cultivar tissue. Can. J. Bot. 54, 814-819.

SHANTZ E.M. & STEWARD F.C. 1955 The identification of compound A from coconut milk as 1,3-diphenylurea. J. Am. Chem. Soc. 77, 6351-6353.

SHORT K.C. & TORREY J.G. 1972a Cytokinins in seedling roots of pea. Plant Physiol. 49, 155-160.

SINGHA S. & BHATIA S.K. 1988 Shoot proliferation of pear cultures on medium containing thidiazuron and benzylamino purine. HortScience 23, 803.

SKENE K.G.M. 1972a Cytokinin like properties of the systemic fungicide benomyl. J. Hortic. Sci. 47, 179-182.

SKENE K.G.M. 1972b Cytokinins in the bleeding sap of the grape vine. pp. 476-483 in Carr D.J. (ed.) 1972 (q.v.).

SKOOG F. (ed.) 1980 *Plant Growth Substances*. Proc. 10th Int. Conf. Plant Growth Substances. Madison, Wisconsin, 1979. Springer-Verlag, Berlin, Heidelberg.

SKOOG F. & MILLER C.O. 1957 Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp. Soc. Exp. Biol. 11, 118-131.

SKOOG F. & TSUI C. 1948 Chemical control of growth and bud formation in tobacco stem segments and callus cultured *in vitro*. Am. J. Bot. 35, 782-787.

SKOOG F., SCHMITZ R.Y., BOCK R.M. & HECHT S.M. 1973 Cytokinin antagonists: synthesis and physiological effects of 7-substituted 3-methyepyrazolo (4;3-d) pyrimidines. Phytochemistry 12, 25-37.

SKOOG F., SCHMITZ R.Y., HECHT S.M. & FRYE R.B. 1975 Anticytokinin activity of substituted pyrrolo 2,3-d pyrimidines. Proc. Nat. Acad. Sci. U.S.A. 72, 3508-3512.

SKOOG F., STRONG F.M. & MILLER C.O. 1965 Cytokinins. Science 148, 532-533.

SOMERS D.A., GEGENBACH B.G., BIESBOER D.D., HACKETT W.P. & GREEN C.E. (ed.) 1986 Abstracts VI Int. Cong. Plant Tissue and Cell Culture. Internat. Assoc. Plant Tiss. Cult. Minneapolis, Minn.

START N.G. & CUMMING B.G. 1976 *In vitro* propagation of *Saintpaulia ionantha* Wendl. HortScience 11, 204-206.

STIRK W.A., STRNAD M., NOVAK O. & VAN STADEN J. 2003 Cytokinins in macroalgae. Plant Growth Regul. 41, 13-24.

SUN J., NIU Q-W., TARKOWSKI P., ZHENG B., TARKOWSKA D., SANDBERG G., CHUA N-H. & ZUO J. 2003 The *Arabidopsis* AtlPT8/PGA22 gene encodes an isopentenyl transferase that is involved in *de novo* cytokinin biosynthesis. Plant Physiol. 131, 167-176

SUNDERLAND N. & WELLS B. 1968 Plastid structure and development in green callus tissues of *Oxalis dispar*. Ann. Bot. 32, 327-346.

SUTTLE J.C. 1984 Effect of the defoliant thidiazuron on ethylene evolution from mung bean hypocotyl segments. Plant Physiol. 75, 902-907.

SUTTLE J.C. 1985 Involvement of ethylene in the action of the defoliant thidiazuron. Plant Physiol. 78, 272-276.

SUZUKI T., MIWA K., ISHIKAWA K., YAMADA H., AIBA H., MIZUNO T. 2001 The *Arabidopsis* sensor kinase, AHK4, can respond to cytokinin. Plant Cell Physiol. 42, 107-113

SYONO K. & FURUYA T. 1972 Effects of cytokinins on the auxin content of tobacco calluses. Plant Cell Physiol. 13, 843-856.

TAKAHASHI S., SHUDO K., OKAMOTO T., YAMADA K. & ISOGAI Y. 1978 Cytokinin activity of N-phenyl-N′-(4-pyridyl) urea derivatives. Phytochemistry 17, 1201-1207.

TANIMOTO S. & HARADA H. 1982 Effects of cytokinin and anticytokinin on the initial stage of adventitious bud differentiation in the epidermis of *Torenia* stem segments. Plant Cell Physiol. 23, 1371-1376.

TAYLOR N.J., STIRK W.A. & VAN STADEN J. 2003 The elusive cytokinin biosynthetic pathway. S. Afr. J. Bot. 69, 269-281.

TEPFER D.A & FOSKET D.E. 1978 Hormone mediated translational control of protein synthesis in cultured cells of *Glycine max*. Dev. Biol. 62, 486-497.

TERRINE C., DOREE M., GUERN J. & HALL R.H. 1972 Uptake of cytokinins by *Acer pseudoplatanus* cells: enzymes of the adenosine deamininase type as possible regulators of the cytokinin level inside the cell. pp. 467-475 in Carr D.J. (ed.) 1972 (q.v.)

THOMAS J.C. & KATTERMAN F.R. 1986 Cytokinin activity induced by thidiazuron. Plant Physiol. 81, 681-683.

THORPE T.A. & MEIER D.D. 1972 Starch metabolism, repiration and shoot formation in tobacco callus cultures. Physiol. Plant. 27, 365-369.

THORPE T.A. & MEIER D.D. 1974 Starch metabolism in shoot-forming tobacco callus. J. Exp. Bot. 25, 288-294.

THORPE T.A. & MURASHIGE T. 1968 Starch accumulation in shoot-forming tobacco callus cultures. Science 160, 421-422.

TORREY J.G. 1972 On the initiation of organisation in the root apex. pp. 1-13 in Miller and Kuehnert (eds.) 1972 (q.v.).

VAN DER KRIEKEN W.M., CROES A.F., BARENDSE G.W.M. & WULLEMS G.J. 1988 Uptake and metabolism of benzyladenine in the early stage of flower bud development *in vitro* in tobacco. Physiol. Plant. 74, 113-118.

VAN STADEN J. & CROUCH N.R. 1996 Benzyladenine and derivatives - their significance and interconversion in plants. Plant Growth Regul. 19, 153-175

VAN STADEN J. & DAVEY J.E. 1979 The synthesis, transport and metabolism of endogenous cytokinins. Plant Cell Environ. 2, 93-106.

VAN STADEN J. & DREWES F.E. 1991 The biological activity of cytokinin derivatives in the soybean callus bioassay. Plant Growth Regul. 10, 109-115.

VAN STADEN J. & DREWES F.E. 1992 The stability and metabolism of benzyladenine glucosides in soybean callus. J. Plant Physiol. 140, 92-95.

VAN STADEN J. & MOONEY P.A. 1988 The effect of cytokinin preconditioning on the metabolism of adenine derivatives in soybean callus. J. Plant Physiol. 133, 466-469.

VANKOVÁ R., GAUDINOVÁ A., KAMÍNEK M. & EDER J. 1992 The effect of interaction of synthetic cytokinin and auxin on production of natural cytokinins by immobilized tobacco cells. pp. 47-51 in Kamínek M., Mok D.W.S., Zazímalová E., (eds). *Physiology and Biochemistry of Cytokinins in Plants*. SPB Academic Publishing, The Hague, The Netherlands,.

VESELY J., HAVLICEK L., STRNAD M., BLOW J.J., DONELLA-DEANA A., PINNA L., LETHAM D.S., KATO J., DETIVAUD L. LECLERC S. & MEIJER L. 1994 Inhibition of cyclin-dependent kinases by purine analogues. Eur. J. Biochem. 224, 771-786.

VIEITEZ A.M. & VIEITEZ M.L. 1980b Culture of chestnut shoots from buds *in vitro*. Plant Physiol. 55, 83-84.

WELSH J. & SINK K.C. 1981 Morphogenic responses of *Browallia* leaf sections and callus. Ann. Bot. 48 583-590.

WENCK A.R., CONGER B.V., TRIGIANO R.N. & SAMS C.E. 1988 Inhibition of somatic embryogenesis in Orchardgrass by endogenous cytokinins. Plant Physiol. 88, 990-992.

WILCOX E.J., SELBY C. & WAIN R.L. 1978 Studies on plant growth-regulating substances. L. The cytokinin activity of some substituted benzyloxypurines. Ann. Appl. Biol. 88, 439-444.

WILCOX E.J., SELBY C. & WAIN R.L. 1981 The cytokinin activities of 6-α-alkylbenzyloxy-purines. Ann. Appl. Biol. 97, 221-226.

WOOD H.N., STERNER R., ALVES L.M. & BASILE D.V. 1990 Auxin-phorbol ester: an example of a two-stage initiation-promotion system mediating cell proliferation in plants. In Vitro Cell. Dev. Biol.-Plant 26, 1125-1127.

XIANG-CAN Z., JONES D.A. & KERR A. 1989 Regeneration of shoots on root explants of flax. Ann. Bot. 63, 297-299.

ZAZÍMALOVÁ E., BREZINOVÁ A., HOLIK, J. & OPATRNY, Z. 1996 Partial auxin deprivation affects endogenous cytokinins in an auxin-dependent, cytokinin-independent tobacco cell strain. Plant Cell Rep. 16, 76-79

ZAZÍMALOVÁ E., BREZINOVÁ, A., MOTYKA, V. & KAMÍNEK, M. 1999 Control of cytokinin biosynthesis and metabolism pp. 141-160 in Hooykaas P, Hall M.A., Libbenga K.R., (eds) 1999. *Biochemistry and Molecular Biology of Plant Hormones,* Ser. New comprehensive biochemistry, Elsevier.

ZIMMER K. & PIEPER W. 1975 Weitere untersuchungen zur kultur *in vitro* von *Aechmea*. Gartenbauwiss. 40, 129-132.

ZIMMER K. & PIEPER W. 1976 Methods and problems of clonal propagation of bromeliads *in vitro*. Acta Hortic. 64, 25-29.

ZIV M., HALEVY H. & SHILO R. 1970 Organs and plantlets regeneration of gladiolus through tissue culture. Ann. Bot. 34, 671-676.

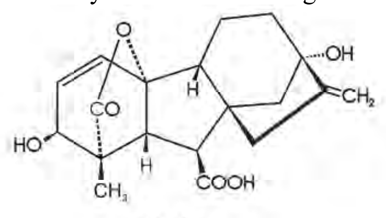
ZUBKO E., ADAMS C.J., MACHÁČKOVÁ I., MALBECK J., SCOLLEN C. & MEYER, P. 2002 Activation tagging identifies a gene from Petunia hybrida responsble for the production of active cytokinins in plants. Plant J. 29, 797-808.

Chapter 7
Plant Growth Regulators III: Gibberellins, Ethylene, Abscisic Acid, their Analogues and Inhibitors; Miscellaneous Compounds

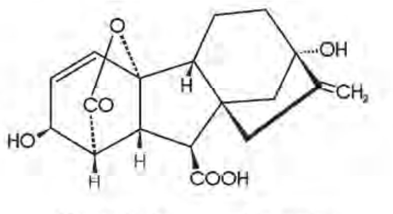
1. GIBBERELLINS

1.1. NATURAL OCCURRENCE AND PHYSIOLOGICAL ACTIVITY

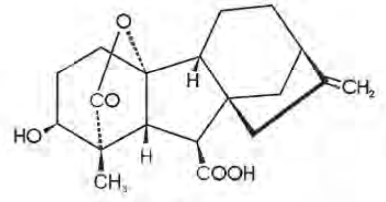
More than 100 members of this group of plant hormones are now known. They all share gibbane ring structures and are either dicarboxylic (C_{20}) or monocarboxylic (C_{19}), they have all been assigned 'gibberellin numbers' (GA_x) and are usually referred to by these rather than by conventional chemical nomenclature. No plant appears to possess all of the gibberellins, some have only been found in fungi and some only in higher plants; nor are the various gibberellins equally active, some are precursors and some catabolites of active gibberellins. GA_1 (1) is the most active gibberellin in the promotion of cell elongation. Very few gibberellins are available commercially and GA_3 (2) or a mixture of GA_4 (3) and GA_7 (4) have been used most frequently in plant culture.



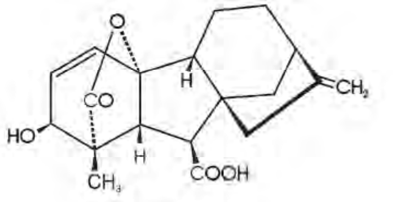
(1) GA_1



(2) GA_3 (gibberellic acid)



(3) GA_4



(4) GA_7

Gibberellins are involved in a wide range of developmental responses. These include promotion of elongation in stems and grass leaves, due in part to activation of the intercalary meristem. Another important role of gibberellins is the induction of hydrolytic enzymes such as α-amylase and protease in the seeds of grasses and cereals, hence facilitating endosperm mobilisation. Other roles in some plants include the promotion of seed germination, bolting of rosette plants, sex determination, fruit development and the control of juvenility.

1.1.1. Biosynthesis and mode of action

The biosynthetic pathway(s) for gibberellins are very complex (Fig. 7.1). All start from isopentenyl diphosphate which in response to soluble cyclases produces *ent*-kaurene(in plastids). Membrane mon-oxygenases then convert this to the common precursor GA_{12} aldehyde which - in a series of steps involving hydroxylases and oxidases - yields the active gibberellins (see Hedden, 1999). Very little is known about the early steps in gibberellin signal transduction. It is clear however that later steps involve selective gene transcription and *de novo* protein synthesis.

1.1.2. Inhibitors of biosynthesis and action

Because very little is known about the mode of action of gibberellins it is doubtful that the action of any of the substances known to affect developmental responses involving these growth regulators is due to effects early in signal transduction. On the other hand, much is known about a wide range of synthetic

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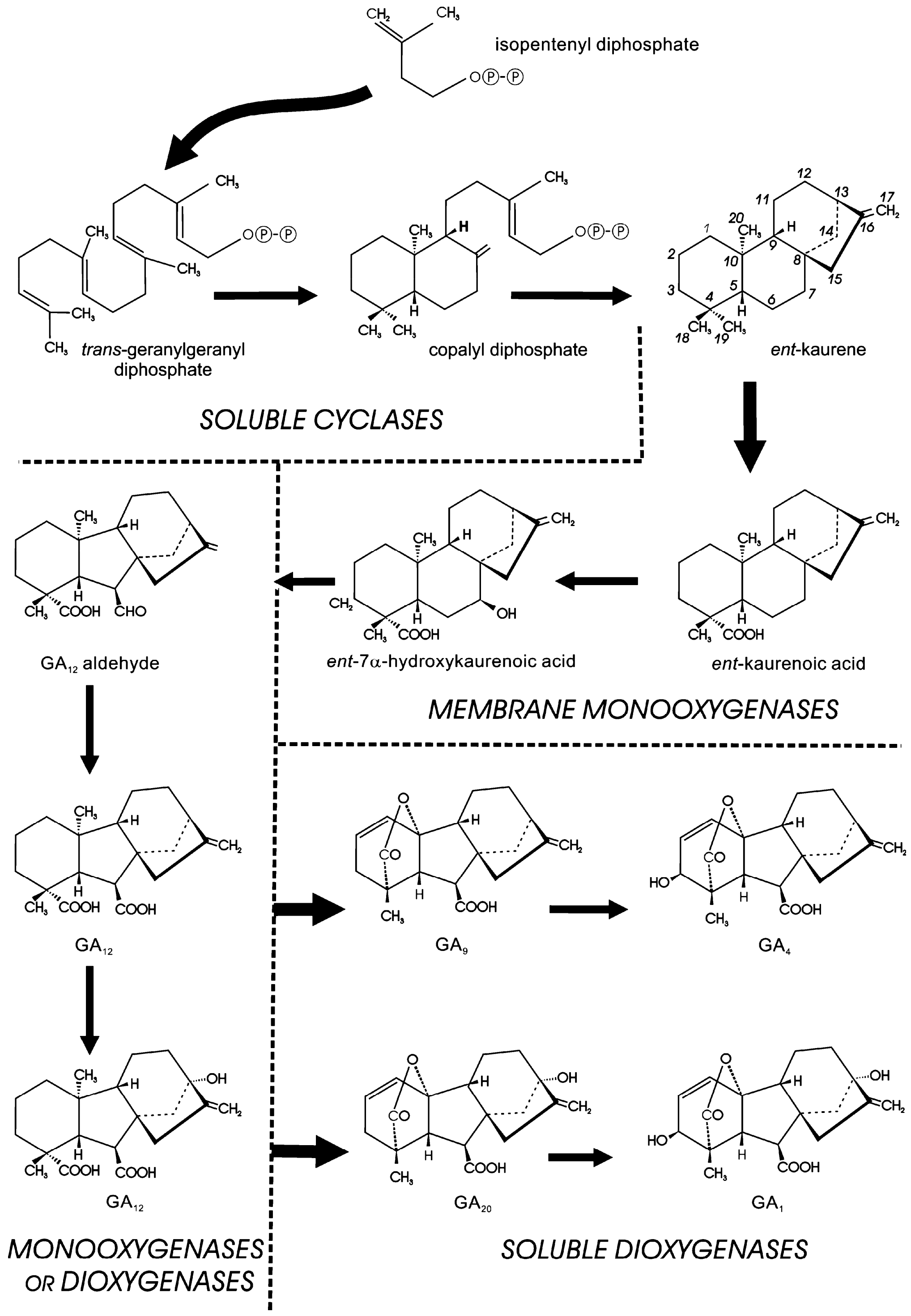
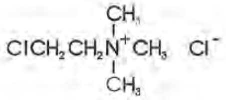


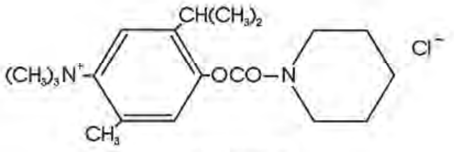
Fig. 7.1 Pathways of gibberellin biosynthesis

substances, often called 'antigibberellins', which act by blocking biosynthetic pathways.

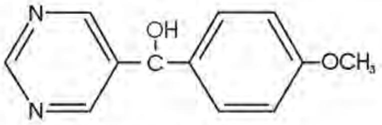
These were in general developed to achieve desirable agricultural outcomes - for example dwarfing of cereals to prevent lodging.These substances fall into four categories (see Rademacher, 2000). A number of quaternary ammonium, phosphonium and sulphonium salts act by inhibiting the cyclisation process. Examples of this type are chlormequat chloride (CCC) (5) and AMO 1618 (6). Certain heterocyclic nitrogen-containing compounds such as ancymidol (7), paclobutrazol (8), uniconazole-P (9) and tetcyclasis (10) appear to act by inhibiting *ent*-kaurene oxidase.



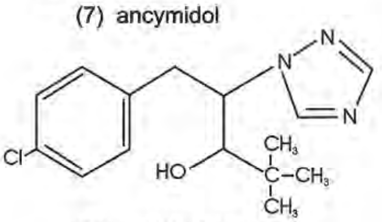
(5) CCC
[chlormequat (chloride)]



(6) AMO1618

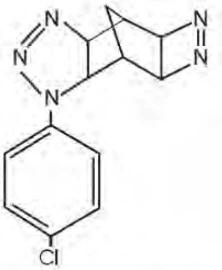


(7) ancymidol



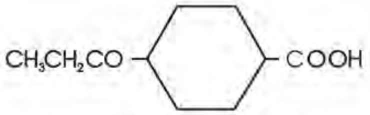
(8) paclobutrazol

(9) uniconazole-P

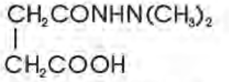


(10) tetcyclasis

A further group of inhibitors are the acylcyclohexanedione derivatives, for example prohexadione (11) and daminozide (12), which affect the later steps of gibberellin biosynthesis involving hydroxylases. While the inhibitors may be useful tools, it should be noted that none are absolutely specific and may affect other biosynthetic pathways such as those for sterols and abscisic acid. Lastly, the inhibitor, 16,17-dihydro GA_5 (13) and related structures appear to act by mimicking the natural substrates.



(11) prohexadione



(12) daminozide

(13) 16,17 - dihydro-GA_5

1.2. EFFECTS OF GIBBERELLINS ON TISSUE CULTURES

Plant tissue cultures can generally be induced to grow and differentiate without gibberellins, although GA_3 acid may become an essential ingredient of media for culturing cells at low densities (Stuart and Street, 1971). When GA_3 is added to culture media, it often produces effects, which are of a similar nature to those of auxins. High concentrations of GA_3 (e.g. greater than ca. 5 μM; 1-8 mg/l) induce the growth of undifferentiated callus cells (Schroeder and Spector, 1957; Murashige, 1964; Mehra and Mehra, 1972; Altman and Goren, 1974; Beasley, 1977; Gautam *et al.,* 1983), and can promote the growth of callus in combination with auxin and low concentrations of cytokinin (Engelke *et al.,* 1973). Growth of *Solanum xanthocarpum* callus was, however, reported to be inhibited by 2 mg/l GA_3 (Rao and Narayanaswamy, 1968). GA_3 can also enhance the growth of cells in suspension cultures [e.g. cotton, (Davidonis, 1990)].

A growth factor, which is probably a gibberellin, is produced by the germinating embyros of some plant species and must be transmitted to the

endosperm before this tissue will proliferate to form callus in culture (Brown *et al.,* 1970; Johri and Bhojwani, 1977). Where the presence of both auxin and cytokinin in a growth medium leads to rapid callusing of the cut surfaces of an explant, the further addition of a small amount of GA_3 (e.g. 0.1 mg/l), or the replacement of auxin by GA_3, usually inhibits callus growth (Sangwan *et al.,* 1976; Kartha *et al.,* 1977).

1.2.1. Morphogenesis

When GA_3 is added to plant tissue culture media, it often diminishes or prevents the formation of adventitious roots, shoots or somatic embryos. Thus, the prior treatment of callus (Murashige, 1964; Sankhla *et al.,* 1994) or explants (Heide, 1969) with GA_3, or the addition of GA_3 to the medium together with auxin and cytokinin at concentrations which would normally promote morphogenesis, is usually inhibitory [e.g. to shoot formation from callus (Murashige, 1961; Rubluo *et al.,* 1984) or embryogenesis (see later)].

1.2.2. Adventitious shoot formation

In tobacco callus, GA_3 was particularly inhibitory to shoot formation if present at the time of meristemoid formation, and more repressive during dark incubation than in the light (Thorpe and Meier, 1973). The inhibition has been shown not to be irreversible, but to persist through at least two subcultures on a GA_3-free medium. Gibberellins other than GA_3 had the same effect (Murashige, 1964). However, the addition of 1.5-3.0 μM GA_3 to the medium (which also contained 2-iP and IBA) supporting *Apios americana* internode explants, increased the number of shoots formed from primary callus on each explant, but the number of explants which produced shoots was less (Wickremesinhe *et al.,* 1990).

In some plants, GA_3 alone can induce adventitious shoot formation, e.g. from *Ranunculus scleratus* callus (Konar and Konar, 1973). The compound can also act as a replacement for auxin in the induction of shoot formation (Sekioka and Tanaka, 1981); a precise gibberellin/cytokinin ratio (instead of auxin/cytokinin) may then be required (Pillai and Hildebrandt, 1969; Engelke *et al.,* 1973). Alternatively, gibberellin may simply increase the number of organs formed; shoot regeneration from *Rosa hybrida* callus could be induced by 1-5 mg/l BAP (depending on cv.), but adding 0.3-1 mg/l GA_3 increased the number of shoots produced (Valles and Boxus, 1987 a,b). Adding 5-50 μM GA_3 to the medium (in conjuction with IBA and BAP) enhanced the formation of vegetative shoots from inflorescence segments of sugarbeet (Coumans-Gilles *et al.,* 1981; Coumans *et al.,* 1982).

The combination of GA_3 and cytokinin is generally less satisfactory for the induction of shoots than that of auxin and cytokinin, and tissues may have to be transferred to a medium lacking GA_3 for further bud development to occur (Kartha *et al.,* 1974a). Shoots formed in the presence of GA_3 may be pale and abnormal (see Chapter 13).

GA_3 can also prevent direct shoot regeneration, for example in:

- *Begonia* leaf discs (Schraudolf and Reinert, 1959: Schott and Schraudolf, 1967: Bigot and Nitsch, 1968; Heide, 1969; Chlyah, 1972)
- *Heloniopsis* leaf segments (Kato and Hongo,1974)
- Sugarbeet floral axillary buds (Coumans-Gilles *et al.,* 1981)

Some of the differences in the inhibition or promotion of adventitious shoot formation by GA_3 might be due to the fact that the compound inhibits meristemoid initiation, but is required for shoot development once meristemoids are formed (Jarret and Hasegawa, 1981). The timing and duration of treatments will therefore strongly influence the results observed.

Many more established plants could be obtained if petiole explants of *Begonia hiemalis* were placed into liquid culture after adventitious shoots had been initiated on an agar medium. The further addition of 30-60 μM (10-20 mg/l) GA_3 increased the number of suitable shoots per explant, but a smaller proportion of the shoots rooted, and their establishment *ex vitro* was not satisfactory (Simmonds and Werry, 1987).

1.2.3. Rhizogenesis

Normally GA_3 inhibits root formation, and the local application of relatively high concentrations (1-10 mg/l) of the compound to the base of cuttings prevents root formation (Brian, 1959), especially if auxins are applied at the same time. Varga and Humphries (1974) showed that although root formation from the base of the petioles of detached *Phaseolus* leaves was inhibited by topical application, it was promoted when GA_3 was applied to leaf laminae. Promotion was especially pronounced when tryptophan (a biochemical precursor of IAA) was placed on the leaf surface at the same time as the GA_3. This, and other evidence, led to their suggesting that promotion of root formation was in

this case due to the gibberellin causing the leaf to produce increased natural auxin, which was transported to the base of the petiole. GA_3 can slightly stimulate root initiation from tomato leaf discs kept in darkness, and Coleman and Greyson (1977a, b) concluded that this is because natural auxin biosynthesis is increased.

Nanda *et al.,* (1972) found that root formation on cuttings of *Ipomoea fistula* was stimulated if they were dipped into GA_3 before being placed in compost. Anand *et al.,* (1972) obtained similar results, but found that pre-treatment with IBA was more effective. GA_3 also improved the rooting of shoots from *Coffea arabica* shoot cultures if a 25-50 mg/l solution was applied directly to shoots at the time they were excised for rooting (Sondahl *et al.,* 1985).

The inhibition of rooting caused by GA_3 is generally magnified in the presence of auxin, and Coleman and Greyson (1977b) proposed that this might be due to an excessive total auxin concentration. This view is supported by results of Rücker (1982) who found that GA_3 was also able to promote direct root formation on leaf fragments of *Digitalis* when applied with low levels of IAA, but was inhibitory when the concentration of IAA was increased.

In some plants however, pre-treating plant material with GA_3 enhances root formation when cuttings are afterwards placed on a root-inducing medium. Rooting can sometimes also be promoted by GA_3 if it is applied to cuttings in the absence of an auxin, and the plant material (or at least the rooting zone) is subsequently kept in darkness. As in caulogenesis, it has been proposed that the effect of gibberellic acid on root formation is dependent on time of application. If GA_3 was applied to *Pinus radiata* cuttings when they were first cut, it inhibited rooting. It did however strongly enhance rooting when given just as there was the first sign of observable root formation, but shortly afterwards it was again inhibitory (Smith and Thorpe, 1975). There are, however, examples of root formation occurring in the presence of gibberellic acid and synthetic auxins:

- The number of roots formed by tomato leaf disc explants treated with 20 mM indole-3-lactic acid was further increased by adding 100 μM GA_3 (Coleman and Greyson, 1977b);
- The inhibition of direct root and shoot formation on isolated leaves of *Begonia rex* by 1-10 mg/l GA_3 was overcome by adding 2,4-D at an equivalent concentration (Schraudolf and Reinert, 1959);
- Direct root formation from *Helianthus tuberosus* rhizome pieces was stimulated when 0.35-3500 mg/l GA_3 was added to NAA (0.2 mg/l) and the explants were kept in the dark, whereas in the light GA_3 was inhibitory (Gautheret, 1969). Six other gibberellins also gave similar results (Tizio *et al.,* 1970).
- GA_3 was able to promote rooting of Prunus shoot cultures at 26ºC in conjunction with the auxin IBA; below this temperature it had no effect (Rosati *et al.,* 1980).

By adding 1 mg/l GA_3 to the growth medium, Nemeth (1979) increased the root formation, which was unusually induced on *in vitro* shoots of *Prunus myrobalan* by the cytokinin BAP. GA_3 in combination with indolebutyric acid and benzyladenine promoted rhizogenesis from callus of *Pinus taeda* (Tang and Fan, 1999)

1.2.4. Embryogenesis and embryo development

Although occasionally reported to be promotory (Evans *et al.,* 1976; Lakshmi Sita *et al.,* 1979; Komai *et al.,* 1996), GA_3 has generally been found to inhibit somatic embryo formation (Fujimura and Komamine, 1975; Tisserat and Murashige, 1977b, c; Kochba *et al.,* 1978; Spiegel-Roy and Kochba, 1980, Hutchinson *et al.,* 1997,Tokuji and Kuriyama, 2003, Cheong and Pooler, 2004). Indeed, the addition of inhibitors of gibberellin biosynthesis increased the number of somatic embryos produced from *Citrus sinensis* callus (Spiegel-Roy and Saad, 1986), although paclobutrazol (an antigibberellin, see above) had little effect in *Pennisetum purpureum* (Rajasekaran *et al.,* 1987). GA_3 has also been shown to antagonise the effect of ABA in promoting embryogenesis (Emons *et al.,* 1993)

The growth ('germination') of preformed somatic embryos of several different species can be stimulated by the incorporation of GA_3 (ca. 0.3-1 mg/l) into the second (post-initiation) medium (e.g. in *Citrus* - Ranga Swamy, 1959, 1961; Kochba *et al.,* 1972, 1974; Button and Kochba, 1977; grapevine - Mullins and Srinivasan, 1976; maize and Guinea grass - Lu and Vasil, 1981, 1982; Lu *et al.,* 1982; Das *et al.,* 1995; *Hordwickia binata* - Ghosh and Sen, 1991; *Dioscorea alata* – Twyford and Mantell, 1996; Kebebew *et al.,* 1998; Canhoto *et al.,* 1999). In some plants, embryo root growth is especially promoted, in others (e.g. *Santalum album* - Bapat and Rao, 1979; *Panax ginseng* - Chang and Hsing, 1980 a,b; Shoyama *et al.,* 1988) shoot regeneration is

stimulated. These results accord with the observations of Noma *et al.,* (1982) that levels of polar gibberellins (GA_3 is of this type) were high in undifferentiated carrot cells and in a non-embryogenic strain. When somatic embryos were initiated they contained low levels of polar gibberellins and metabolised these compounds rapidly. In *Citrus* and *Duboisia* (Nwankwo and Krikorian, 1983), once embryos have started to grow, plantlets need to be transferred to a medium lacking gibberellin, otherwise shoots are weak and spindly. The growth of soybean somatic embryos was promoted by antigibberellins (Gamborg *et al.,* 1983).

1.2.5. Cellular differentiation

GA_3 has little effect on cellular differentiation *in vitro* beyond mediating the action of auxin. In intact plants, xylem formation is observed to be stimulated by treatment with GA_3 or with auxin and GA_3 combined (Wareing, 1958; Bostrack and Struckmeyer, 1967; Dorley and Leyton, 1968). Auxin is now thought to be mainly responsible for the initial differentiation of phloem and xylem elements in tissue cultures (Aloni, 1980), even though some promotion by GA_3 has been reported (Gautheret, 1961; Roberts and Fosket, 1966; Roberts, 1969; Dalessandro, 1973; Phillips and Dodds, 1977).

In the cactus *Opuntia polyacantha*, axillary meristems became leafy shoots on media with 5-10 mg/l of the cytokinin BAP, but produced spines with 20-100 mg/l GA_3. When 5-50 mg/l NAA was used, roots arose from bud trace procambium (Mauseth and Halperin, 1975).

1.2.6. Enzyme activity

Gibberellins influence the biosynthesis or activity of enzymes and can consequently affect the production of secondary plant products *in vitro*, e.g. the synthesis of amaranthine (Laloraya *et al.,* 1976) and anthocyanin (Heinzmann and Seitz, 1977; Mizukami *et al.,* 1988), which are inhibited by the presence of GA_3 in the medium. The failure of carrot cells to produce anthocyanin in these circumstances is apparently due to the absence of a *p*-coumaric acid:CoA ligase isoenzyme (Heinzmann *et al.,* 1977).

1.3. USE IN MERISTEM, SHOOT AND NODE CULTURES

Although GA_3 tends to prevent the formation of organised root and shoot meristems in callus cultures, it may assist the further growth and development of preformed organs. The growth of shoots in meristem and shoot cultures may also be enhanced by its addition.

1.3.1. Meristem cultures

A small amount of GA_3 (typically 0.03-0.1 mg/l) is often added to media for meristem cultures. It may not always be beneficial or absolutely necessary, and there are conflicting results. Mellor and Stace-Smith (1969), for example, found the substance to be without any appreciable effect on meristem tip cultures of potato, but other investigators (e.g. Morel *et al.,* 1968; Pennazio and Redolfi, 1973; Novák *et al.,* 1980) have reported that the addition of low concentrations of GA_3 (e.g. 0.03 mg/l) to media containing auxin and cytokinin, or cytokinin alone, has improved the growth of meristem tips, inhibited callus proliferation, sometimes encouraged the tips to root more freely, and (Marani and Pisi, 1977) increased the proportion of meristems that developed into shoots. Similar effects have been noted in meristem cultures of some other plants (e.g. cassava - Kartha *et al.,* 1974b; Rey and Mroginski, 1978). In some cases there are strong varietal differences; in meristem cultures of sweet potato, GA_3 promoted multiple shoot growth and elongation in cv. Mae de Familia but not in cv. Covacao Alado (Dagnino *et al.,* 1991)

GA_3 does not assist in the establishment of the shoot apices of all plants. It was found to be inhibitory to growth of meristems tips of some kinds of geraniums (Theiler, 1977) and flax (Lane, 1979), and in rose, apices grew rapidly and formed abnormal attenuated leaves, but no roots or new leaves were initiated (Jacobs *et al.,* 1969, 1970; Elliott, 1970).

As mentioned previously, GA_3 frequently inhibits root formation, especially in the presence of auxin. When the compound is used to stimulate the growth of isolated meristems or shoot tips therefore, it may prevent eventual root development (Murashige, 1961; Vine and Jones, 1969; Putz, 1971). Plantlets formed on media containing GA_3 may need to be moved to a medium containing auxin, but no gibberellin, before they can be rooted (Quak, 1977).

1.3.2. Shoot and node cultures

GA_3 is added to the medium, together with auxin and cytokinin, for Stages I and II of shoot cultures of certain plants. In some genera it is effective when auxins are not. At Stage I its presence can improve establishment, for example, De Fossard and de Fossard (1988) found that the addition of GA_3 to the medium was useful to initiate growth in cultures from adult parts of trees of the family Myrtaceae, although thereafter it had a deleterious effect.

1.3.3. Shoot multiplication

At Stage II, GA_3 can enhance growth and/or increase the rate of shoot proliferation; adding 1.5 mM GA_3 to a medium containing 2-iP and IBA increased the number of shoots produced from seedling and tuber-shoot internodes of *Apios americana*, but reduced the number of explants which produced shoots (Wickremesinhe *et al.,* 1990). Addition of GA_3 with benzyladenine caused high frequency bud break and shoot multiplication in apical shoot buds and nodal explants of *Morus cathayana* (Pattnaik and Chand, 1997). Similar results have been obtained with *Ocimum basilicum* (Sahoo *et al.,* 1997).

The benefits of using GA_3 at Stage II often vary between closely related plant genotypes. Thus GA_3 was antagonistic to shoot proliferation in 'McIntosh' apple shoot cultures (Lane, 1978), but was essential for 'Ottawa 3' apple rootstock, where without the addition of 5 mg/l GA_3, shoots had short internodes and small deformed leaves.

Valles and Boxus (1987a,b) found that the addition of some GA_3 (0.1-1.0 mg/l depending on genotype) to a medium containing 1-5 mg/l BAP was essential to obtain shoot multiplication of some *Rosa hybrida* cultivars, and an improved rate of proliferation in others. Cultivar 'Goldy' did not proliferate at all unless 1 mg/l GA_3 was present. Adding 0.7 mg/l GA_3 to 2.3 mg/l BAP increased the rate of proliferation in *Camellia saluensis* and *C. japonica* shoot cultures (Beretta and Eccher, 1987). Substituting IAA (0.2 mg/l) for GA_3 in *Camellia* caused only an increase in shoot length; there was no shoot proliferation.

Shoot cultures of the salt-tolerant plant *Atriplex canescens* were found to remain stunted without the application of GA_3 (Wochok and Sluis, 1980). Addition of the growth regulator to the agar medium was ineffective and so filter-sterilised solution was flooded onto the plates on which the explants were growing and then decanted away after a few seconds, leaving a residue. This treatment had the effect of first promoting the multiplication of adventitious shoots, and then afterwards stimulating their elongation. Similarly, a combination of GA_3 (0.01-0.1 mg/l) and kinetin (0.5-5 mg/l) with no auxin has been found to provide a highly effective growth regulator treatment for the rapid increase of potato shoots in shaken liquid cultures (Goodwin *et al.,* 1980).

Without the addition of 1 mg/l GA_3 to 1 mg/l IBA and 0.2 mg/l BAP at the multiplication stage, shoot cultures of *Humulus lupulus* produced masses of callus at the basal ends of shoots (Vine and Jones, 1969). Necrosis in *Rosa hybrida* shoot cultures was prevented by adding 0.1 mg/l GA_3 to the proliferation medium (Valles and Boxus, 1987).

1.3.4. Shoot elongation

Shoots are occasionally treated with GA_3 to increase the length of shoots during multiplication; or prior to rooting, when it is usually applied at a special elongation stage (Stage IIIa), after shoot multiplication and before shoots are harvested (see Chapter 12). Treatment may be beneficial where a high level of cytokinin has resulted in many short shoots (e.g. in rose - Valles and Boxus, 1987 a,b). Some examples of successful use are (mg/l):

- *Malus domestica*, 0.1 (Jones *et al.,* 1977, 1979) ; 5.0 (Pua *et al.,* 1983)
- *Malus prunifolia*, 0.1 (Aldwinckle and Gustafson, 1981)
- *Rubus caesius*, 0.1 (Babic and Neskovic, 1984)
- *Simmondsia chinensis*, 2.0 (Jacoboni and Standardi, 1987)
- *Fragaria ananassa*, 0.1 (Boxus, 1974a, b; Boxus *et al.,* 1977)
- *Ficus benjamina*, 0.5 (Delamomarco and Picazo, 1994)
- *Acacia sinuata*, 0.35 (Vengadesan *et al.,* 2000, 2003)

1.3.5. Apical integrity

Cytokinin treatments, used to promote axillary shoot proliferation, can cause shoots to develop with more than one apical meristem (see Chapter 10). In strawberry, GA_3 may help to preserve the integrity of apical buds during shoot culture. Anderson *et al.,* (1982) showed that 0.1 mg/l GA_3 largely eliminated the formation of abnormal multi-apexed plants, which was caused by combinations of BAP and IBA favourable to rapid shoot proliferation.

1.3.6. Detrimental effects

In most plants, the use of gibberellin in shoot culture media is detrimental, producing elongated shoots with narrow leaves. Such was the effect on *Duboisia myoporoides* shoots when 0.1-0.3 mg/l GA_3 was introduced into MS medium containing 3 mg/l BAP and 1 mg/l IAA (Kukreja and Mathur, 1985). In tree species of the Myrtaceae, adding GA_3 to Stage II cultures caused too much shoot elongation, the formation of narrow leaves, and the production of callus at the base of axillary branches (De Fossard

and De Fossard, 1988). GA_3 inhibited bud formation in *Thuja plicata* shoot cultures (Coleman and Thorpe, 1977) and caused shoot tip explants of *Acer freemanii* to develop massive basal callus (Kerns and Meyer, 1986). Shoots treated with GA_3 may be difficult to root.

1.3.7. Stage IV treatments

Plantlets of some woody species may become dormant after being transplanted to soil and fail to grow properly unless subjected to a period of cold, or treated with GA_3. Biosynthesis of gibberellins is often promoted by cold treatment of plant material. Cold treatment of cultured shoots is necessary to promote bulb formation in *Tulipa*, but can be replaced by soaking shoots in 1 mg/l GA_3 for 15 h (Wright *et al.,* 1982; Rice *et al.,* 1983).

1.4. 'ANTIGIBBERELLINS' AND GROWTH RETARDANTS

As exogenous GA_3 is frequently inhibitory to *in vitro* growth and development, chemicals capable of blocking the biosynthesis or action of endogenous gibberellin may have promotory effects on cultures in which natural gibberellin levels are supra-optimal. Although chemicals with both of these kinds of activity are conveniently called anti-gibberellins, those which interfere with gibberellin biosynthesis (which is by far the majority) should not strictly be grouped under this heading, because their effects on plants can usually be reversed by applications of a gibberellin such as GA_3. The nature of these compounds and their mode of action has been described earlier in this chapter.

1.4.1. Onium compounds

The most commonly used of these substances are chlormequat chloride (CCC) and Amo1618. CCC can stimulate embryogenesis in species such as *Citrus* where the process is inhibited by GA_3 (Kochba *et al.,* 1978; Spiegel-Roy and Kochba, 1980). In *Ranunculus sceleratus*, direct shoot formation from hypocotyl segments (which was stimulated by GA_3) was reduced by CCC (Konar and Konar, 1973). Some workers have added small amounts (0.1mg/l) of CCC to their culture medium in the belief that this improves morphogenesis (Blackmon *et al.,* 1981a,b; Blackmon and Reynolds, 1982; Reynolds *et al.,* 1980). De Langhe and De Bruijne, (1976) showed that in tomato, pretreating plants with CCC before explants were removed, stimulated shoot formation in culture. The treatment also changed the auxin/cytokinin balance necessary to achieve shoot formation.

1.4.2. Heterocyclic compounds

Most work with these compounds has centred on paclobutrazol and to a lesser extent ancymidol and uniconazole. All the members are potent inhibitors of gibberellin biosynthesis but they also interfere with the synthesis of abscisic acid and sterols (Davis *et al.,* 1988; Yates *et al.,* 1993). Like other gibberellins they can stimulate embryogenesis where it is inhibited by GA_3 (Spiegel-Roy and Saad, 1986; Hutchinson *et al.,* 1997). They have also been shown to be effective in promoting shoot formation and rhizogenesis in a number of systems (*Helianthus annuus* - Fiore *et al.,* 1997; *Populus tremula* - Vinocur *et al.,* 2000; carnations - Sankhla *et al.,* 1994). Ancymidol has been shown to enhance tuber formation in single node sections from potato plantlets (Levy *et al.,* 1993) and microtuber formation on propagated plantlets (Alchanatis *et al.,* 1994). There is also some evidence that these compounds can improve acclimatization and tolerance to desiccation in plantlets or rooted shoots (Gavidia *et al.,* 1997; Panaia *et al.,* 2000).

1.4.3. Acylhexanediones

Most work in the past has used daminozide - for example in stimulating embryogenesis (Kochba *et al.,* 1978; Spiegel-Roy and Kochba, 1980; Moore, 1985) and decreasing shoot elongation (Stimart, 1986). The newer members of this group - such as prohexadione (11)- have mainly been investigated in the context of intact plants. However, prohexadione does appear to have effects on a greater range of species than daminozide - for example in terms of the retardation of shoot growth (Brown *et al.,* 1997). In hypocotyl explants of *Albizzia julibrissin*, prohexadione was much more effective than either paclobutrazol or uniconazole in promoting adventitious shoot formation (Sankhla *et al.,* 1993).

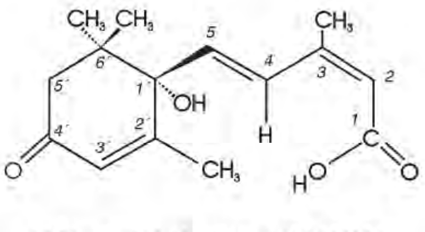
However, the effects of these inhibitors, like those of many others, are not entirely predictable. Thus, stem elongation and flowering of *Matthiola incanum* was promoted **both** by GAs **and** by acylhexanediones (Hisamatsu *et al.,* 2000) and in intact epicotyls of *Vigna sinensis,* LAB 198 999 (another acylhexanedione) inhibited elongation, whereas in debladed seedlings and explants, promotion was observed (Martinez-Garcia and Garcia-Martinez, 1992).

The gibberellin analogues such as 16,17-dihydro GA_5 do not seem to have attracted much attention from workers in the field, probably because of difficulties in obtaining supplies of the compounds.

2. ABSCISIC ACID

2.1. OCCURRENCE AND ACTIVITY OF ABSCISIC ACID

Abscisic acid (ABA) (14) is another naturally-occurring growth substance. It is a 15-carbon acid; four stereoisomers exist, differing in the orientation of the carboxyl group and the attachment of the sidechain to the ring. The naturally-occurring form is *S*-(+)-ABA. Commercially available abscisic acid is a mixture of isomers. ABA appears to be produced as a cleavage product of certain carotenoids - xanthophylls - yielding xanthoxin, which is converted to ABA aldehyde and hence to ABA (Zeevart, 1999)(Fig. 7.2). It has long been known that dehydration of plant tissue leads to increased biosynthesis of ABA (Wright and Hiron, 1969); however, it is now well-established that a number of other environmental factors - low (Vernieri *et al.,* 1991) and high temperatures (Daie and Campbell, 1981), salinity (Kefu *et al.,* 1991) and flooding (Jackson, 1991) - can also produce the same effect.

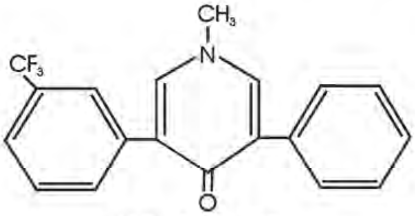


(14) *cis*-(+) abscisic acid (ABA)

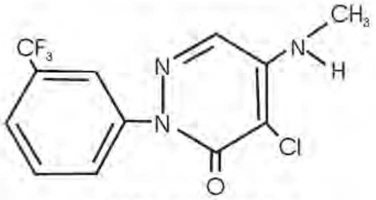
ABA catabolism is complex involving either oxidation/reduction - to phaseic acid and dihydrophaseic acid - or conjugation to produce the glucose ester or glucoside (Zeevart, 1999).

Biosynthesis occurs in plastids (especially chloroplasts) (Milborrow, 1974). The herbicide 'fluridone' (15), which inhibits the natural production of carotenoids (Bartels and Watson, 1978), can prevent ABA biosynthesis (Moore and Smith, 1984). Norflurazon (16) produces the same effect.

Abscisic acid is found ubiquitously in plants and is the most commonly identified of a number of other structurally related natural compounds, which have plant growth regulatory activity. It has often been regarded as being a plant growth inhibitor, partly because of its early history, which involved studies on bud dormancy and abscission. However, ABA has many roles in plants, such as the regulation of stomatal closure, control of water and ion uptake by roots, and of leaf abscission and senescence and hence, like other hormones has multifaceted effects. In tissue cultures, it sometimes promotes morphogenesis or growth. The quantity present in plant cultures can be determined by gas chromatography mass spectrometry or ELISA, following purification by high-pressure liquid chromatography (Ryu *et al.,* 1988). Uptake of ABA into tissues appears to be by simple diffusion of the undissociated molecule, the anions being trapped upon entry into cells. It is decreased at pH levels above 5.5 (Minocha and Nissen, 1985; Patel *et al.,* 1986).



(15) fluridone



(16) norflurazon

The early stages of ABA action probably follow the same types of mechanism as those for other hormones that is, transduction chains leading to changed transcription and translation patterns, or effects on transmembrane ion pumps (as in the case of stomata) (see Assman and Armstrong, 1999; Busk *et al.,* 1999). More specifically, ABA has been shown to control the expression of genes specific to embryo development and maturation. Thus, using ABA-deficient and ABA-insensitive *Arabidopsis* mutants ABA has been shown to control genes for both LEA (late embryogenesis abundant) and storage proteins (see Dodeman *et al.,* 1997).

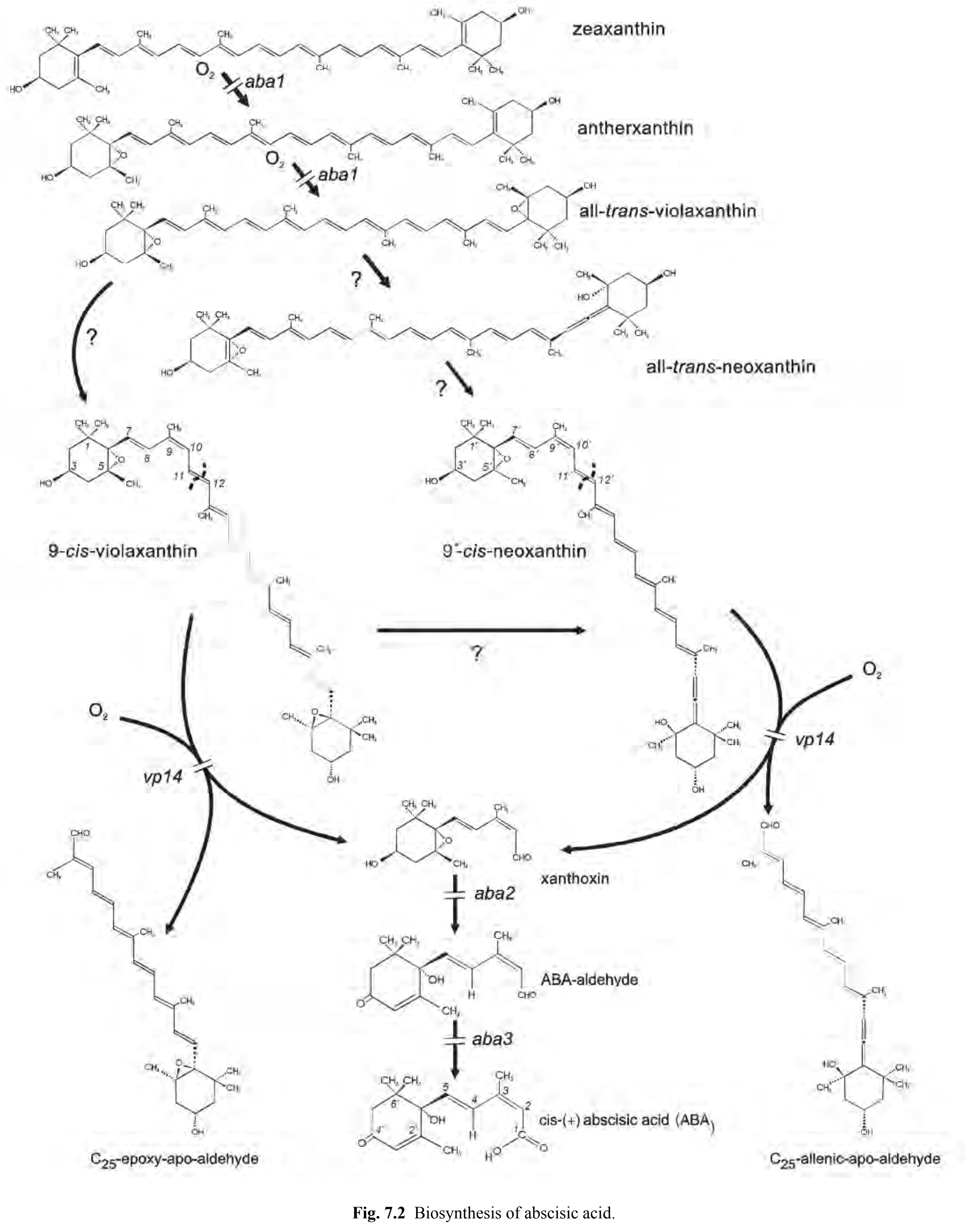


Fig. 7.2 Biosynthesis of abscisic acid.

At another level, some of the effects of ABA lie in the hormone antagonising or modifying the effects of other hormones, notably cytokinins and gibberellins, but also auxins. For example, Charriere *et al.,* (1999) have suggested that the effect of ABA on morphogenesis in zygotic embryos of *Helianthus annuus* is indirect and due to a modification of auxin levels.

2.2. ABA IN TISSUE CULTURES

2.2.1. Effects On Callus Growth

ABA has usually been found to be inhibitory to callus growth. Sankhla and Sankhla (1968) reported that 1 mg/l was markedly inhibitory to *Ipomoea* callus, but generally, concentrations between 5 and 50 mg/l appear to be necessary to cause a 50% growth inhibition of cell growth (Li *et al.,* 1970; Gamborg and LaRue, 1971; Taylor and Burden, 1972). However, several independent instances have been cited where ABA has been capable of stimulating callus growth, for example:

- On hypocotyl explants of *Cryptomeria* (a gymnosperm tree), 1 mg/l ABA in the culture medium had the same effect as 10 mg/l of the cytokinin BAP in stimulating internal callus or adventitious bud formation (Isikawa, 1974).
- On explants consisting of pieces of *Citrus* leaf and stem, 0.3-2.6 mg/l ABA (1-10 μM) stimulated callus formation specifically on the abscission zone between the petiole and the stem (Altman and Goren, 1971).
- In soybean, ABA stimulated callus growth when 0.05 or 0.5 mg/l kinetin was present in the medium. At lower kinetin levels ABA had no effect or was inhibitory (Blumenfeld and Gazit, 1970);
- Haploid tobacco callus was increased in growth by 0.1 mg/l ABA. At 10 mg/l the regulator was completely inhibitory (Kochhar, 1980).
- In excised hypocotyls of carrot ABA in the culture medium promoted callus induction and proliferation in the tissue (Jimenez and Bangerth, 2001).

These responses are usually only obtained with relatively low ABA concentrations: higher rates bring about corresponding decreases in the weight of callus produced (Torrizo and Zapata, 1986). This probably indicates that the concentrations inhibiting callus growth (which have been most commonly used) are unphysiological.

2.2.2. Effects on morphogenesis

Adventitious shoots and roots. ABA has been observed to influence morphogenesis in a number of plants. The first report was by Heide (1968) who found that shoot bud formation on isolated *Begonia cheimantha* leaves (not in aseptic culture) was enhanced when the leaves were treated with abscisic acid, and inhibited when either auxin or gibberellin was applied. Seasonal variation in the capacity of *Begonia* leaves to produce shoot buds was thought to be associated with variation in endogenous ABA levels. Shepard (1980) found that adding ABA to the growth medium (0.05-0.2 mg/l, depending on variety), caused morphogenesis to occur more rapidly than it otherwise would from potato callus. Natural ABA levels have been noted to correspond to the maturation state of tissues (Tanimoto *et al.,* 1985). Adding 100 mg/l ABA to the medium, on which internode segments of *Torenia* were cultured, stimulated the production of flower buds on previously vegetative explants. The greatest amount of flowering occurred when the ABA within the tissues (i.e. the sum of endogenous and exogenous sources) was between 16 and 20 mg/g. Hooker and Thorpe (1998) using cultured tomato roots observed that ABA at concentrations of 0.2 mg/l and above inhibited lateral root initiation and emergence, whereas its putative biosynthesis inhibitor fluridone (15) enhanced the formation of lateral roots. Hartung and Abou-Mandour (1996) observed that regenerates of *Ruta graveolens* only survive transplant shock if pretreated with 2.5 mg/l ABA, which they attributed to a stimulatory effect on lateral root and root hair formation.

Embryogenesis. ABA is essential for the normal growth of somatic embryos and only in its presence do they closely resemble zygotic embryos in their development and structure. Manipulation of endogenous and/or exogenous ABA levels increases the frequency of embryos reaching maturity and can assist the handling of the large populations of somatic embryos which can be required for mass propagation (Ammirato, 1988).

The slight check to embryo development caused by 0.03 mg/l ABA was found by Ammirato (1973, 1974) to be associated with the elimination of abnormal forms of embryos (such as embryos with fused or multiple cotyledons, mature leaves in place of cotyledons, or other accessory structures) that were formed in caraway (*Carum*) suspension cultures, especially in the light. ABA treatment also prevented

the formation of accessory embryos in this tissue, which were otherwise developed on somatic embryos already in existence. The effectiveness of ABA in decreasing the proportion of abnormal embryos and assisting and synchronising embryo maturation (conversion) has since been confirmed in other species (e.g. Kamada and Harada, 1981; Dunstan *et al.,* 1988; Krogstrup *et al.,* 1988; Capuana and Debergh, 1997; Castillo *et al.,* 1998; Cvikrova *et al.,* 1998; Bela and Shetty, 1999; Fernando and Gamage, 2000).

There are also reports of low concentrations of ABA stimulating somatic embryo initiation or embryo growth:

- Embryogenesis was stimulated in growth regulator habituated *Citrus sinensis* (Shamouti orange) callus by 0.01-1 mg/l ABA (Kochba *et al.,* 1978; Spiegel-Roy and Kochba, 1980).
- Embryoids were only developed from suspension cultures of *Pennisetum* when cells were transferred to a medium containing 0.01-0.02 mg/l ABA (Vasil and Vasil, 1981).
- The addition of 0.1-50 mg/l ABA to media containing 2,4-D, adenine and kinetin, increased the number of embryos which grew from the globular stage to the heart-shaped stage in soybean suspension and callus cultures (Phillips and Collins, 1981).
- Adding 0.02-10 mg/l ABA to the medium increased the proportion of *Pennisetum purpureum* leaf explants which produced embryogenic callus, especially from tissues treated with fluridone (0.02-20 mg/l) in which, without ABA, embryogenesis was inhibited (Rajasekaran *et al.,* 1987)
- Transfer of embryo suspensor masses of *Abies fraseri* to media containing 50-80 μm ABA resulted in the formation of cotyledon stage embryos (Guevin and Kirby, 1997)
- ABA and polyethylene glycol are instrumental in the functional development of somatic embryos from conifers (Stassolla *et al.,* 2002)

Several authors have noticed a growth-inhibiting action of exogenous ABA on embryogenesis and on the growth of both somatic and zygotic embryos, but these reports are rare and often describe the effects of high concentrations of the regulant. Although 0.002-2.6 mg/l ABA had little effect during the development of early globular or heart stages of somatic embryos formed in carrot cultures, it decreased the growth at stages beyond this (Fujimura and Komamine, 1975). Embryo formation in *Citrus sinensis* is supressed by 11-21 mg/l ABA (Spiegel-Roy and Kochba, 1980), or by even higher concentrations (Tisserat and Murashige, 1977a).

Pre-formed late stage embryos can be completely arrested in growth by 26.4 mg/l ABA, but remain viable (even though they lack chlorophyll), and will continue to develop and grow when the ABA is removed. ABA treatment might therefore be used to facilitate the storage of embryos needed at some future date for plant propagation (Ammirato, 1974). The inhibition of somatic embryos by ABA is very similar to the inhibition of zygotic embryo growth, which it induces naturally (LePage-Degivry, 1973; Sondheimer *et al.,* 1968); this serves to keep seed embryos dormant, preventing precocious germination.

Seeds of *Dendrocalamus strictus* germinated in the presence of 0.1-1.0 μM ABA, but if 2,4-D was also added, embryogenic callus was produced. However, the number of embryos obtained was finally less than with 2,4-D alone (Rao *et al.,* 1987).

As noted earlier, in many of the cases where ABA has been shown to be inhibitory this is almost certainly due to the use of unphysiological concentrations. However, there is also evidence that part of this may be due to changes in ABA concentrations in cultures during embryogenesis and also differences between types of embryos. Thus, in carrot, Jimenez and Bangerth (2001) showed that there were much higher ABA levels in embryogenic calli compared to non-embryogenic. Similarly, Jimenez and Bangerth (2000), using different callus lines of *Vitis vinifera* showed that there was a strong correlation between high endogenous ABA concentrations and morphogenetic capacity. Similarly, Faure *et al.,* (1998) demonstrated a relationship between precocious germination of somatic embryos of *Vitis vinifera* and low ABA levels, and Kim *et al.,* (1998) showed that while ABA did not promote maturation in embryos of *Larix leptolepis* it was beneficial in reducing precocious germination. It is also clear from the work of Faure *et al.,* (1998) and Gawronska *et al.,* (2000) that somatic and zygotic embryos differ significantly in their ABA contents - generally low in the former and high in the latter - and this is likely to influence the concentration of the hormone at which beneficial effects will be observed.

3. ETHYLENE

The presence of very low concentrations of ethylene (17) in the atmosphere, has long been known to affect plant growth and development. Ethylene concentrations in the gaseous phase are usually expressed as ppm - parts per million (volume per million) - or $\mu l.l^{-1}$ and many ethylene effects show half-maximal responses at $0.1\mu l.l^{-1}$. Since ethylene equilibrates rapidly with the aqueous phase of the cell this corresponds to a concentration of the substance in that phase of about $4.5x10^{-10}M$ at 20^oC.

$CH_2{=}CH_2$

(17) ethylene

It is now known that ethylene is produced by all living plant tissues and regulates their growth. The gas is most obviously involved in fruit ripening, senescence, and the abscission of leaves, but also has many other functions (see Abeles *et al.,* 1992). It is also notable that, depending on the species or the tissues involved, ethylene can have markedly different effects on development. For example, while the gas generally inhibits the growth of dicotyledonous shoots (e.g. peas) it promotes growth in a number of hydrophytes - e.g. rice (Ku *et al.,* 1970; Suge, 1972). Equally, in the light, ethylene can actually promote the growth of *Arabidopsis* seedlings (Smalle *et al.,* 1997).

3.1. ETHYLENE BIOSYNTHESIS

The biosynthetic pathway for ethylene is now well established (see Imaseki, 1999) and is illustrated in Fig. 7.3. Methionine is converted to S-adenosylmethionine, which in turn is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) via the enzyme ACC synthase (ACS); the ACC is then metabolised to ethylene via ACC oxidase (ACO). Methylthioadenosine, the other product of ACS, is recycled to methionine hence conserving sulphur. Unsurprisingly, addition of either methionine, or ACC, to plant tissue of all kinds usually leads to increased rates of ethylene biosynthesis. While, in comparison with other plant hormones, the biosynthetic pathway for ethylene appears to be relatively simple, the issue is complicated because of the existence of isozymes for both the enzymes involved (see Imaseki, 1999). Moreover, these enzymes are controlled differentially. Thus, there are three specific stimuli, which activate gene expression of different ACS isogenes - auxin, wounding or ripening - but this expression in turn can be modulated by other components - ethylene itself, abscisic acid, cytokinin, carbon dioxide and light. Hence, manipulation of tissue or cultures, the environmental conditions and the makeup of media can all influence ethylene production, the final outcome depending on the balance between activation and inactivation of particular genes.

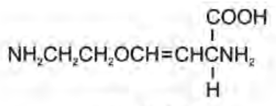
It is also important to note that because ethylene may have either an autocatalytic or an autoinhibitory effect on its own synthesis depending on the tissue or plant species (Vendrell and McGlasson, 1971; Zeroni *et al.,* 1976; McGlasson *et al.,* 1978; Riov and Yang, 1982a; Yang and Hoffman, 1984; Bufler, 1986; Mattoo and White, 1991; McKeon *et al.,* 1995) and that this is receptor-controlled, then the use of receptor-directed inhibitors of ethylene action such as norbornadiene (see below) may in fact result in either increased or decreased biosynthesis of ethylene depending on the tissue type or plant species. Similarly, the ethylene receptor mutants *etr1* and *eti5* of *Arabidopsis* (where ethylene biosynthesis is autoinhibited in wild type) both overproduce the gas. Plants can metabolise ethylene to ethylene oxide via monooxygenase enzymes (Jerie and Hall, 1978); however, with the exception of those from *Vicia faba* and alfalfa, these latter usually show low activity and low specificity and are unlikely to affect internal concentrations markedly (Beyer, 1985).

3.2. INHIBITORS OF BIOSYNTHESIS

A number of compounds or treatments have been shown to inhibit ethylene biosynthesis:

3.2.1. Aminoethoxyvinylglycine (AVG)

AVG (18) is a potent inhibitor of ethylene biosynthesis in plants. It acts by inhibiting ACS (Yang *et al.,* 1980; Kende *et al.,* 1980). In tissue cultures 1-50 µg/l is effective without causing direct toxicity.



(18) AVG

3.2.2. Cobalt ions

A high concentration of cobalt ions inhibits ethylene production in plant tissues (Lau and Yang, 1976) by preventing the conversion of ACC to ethylene. In the presence of 50-500 µM Co^{2+}, ACC levels increase in tissues treated with IAA (Yu and Yang, 1979). Cobalt chloride (10-100 mM) added to rose shoot cultures increased apical dominance (the opposite of the effect produced by low concentrations of ethylene (Kevers *et al.,* 1992).

3.2.3. Anoxia and Oxidation inhibitors

Because oxygen is required for the conversion of ACC to ethylene, anoxia inhibits ethylene production and synthesis may decrease significantly when the partial pressure of oxygen falls below 15 kPa. However, in some plants, such as barley and maize ethylene production is stimulated at 3-5 kPa (Jackson, 1985a, b). Similarly, the effects of a variety of oxidation inhibitors have been assessed in ethylene-forming systems. These have included salicylic acid (19) and acetyl salicylic acid (20) (but also see below), and *n*-propyl gallate (21) as well as uncouplers of oxidative phosphorylation and inhibitors of cytochrome oxidase. However, while they may indeed affect ethylene synthesis their broad-spectrum effects are such that their use for this purpose cannot be recommended, especially since much more specific inhibitors, such as AVG, are available.

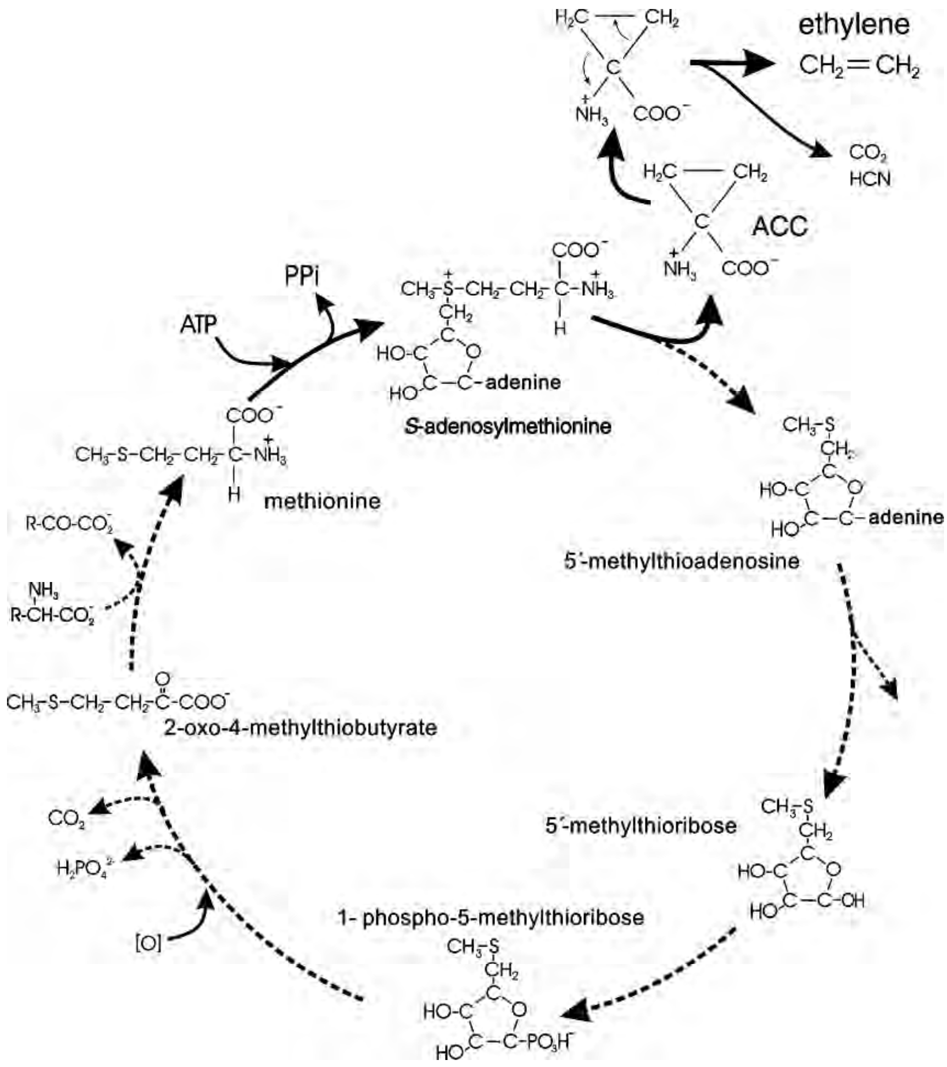
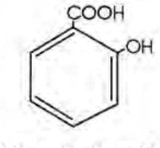
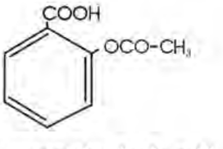


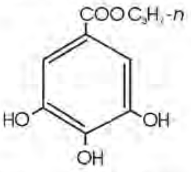
Fig. 7.3 Biosynthesis of ethylene.



(19) salicylic acid



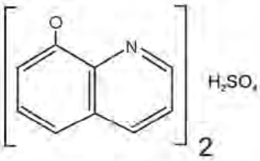
(20) *o*-acetyl-salicylic acid



(21) *n*-propyl gallate

3.2.4. Chelating agents

Chelating agents such as 8-hydroxyquinoline (22) are effective in prolonging the vase life of cut flowers (thereby overcoming ethylene-induced senescence). Their mode of action is unknown, but may be related to their ability to sequester copper ions (see below).



(22) 8-hydroxyquinoline sulphate

3.3. ETHYLENE ACTION

Five partially functionally redundant receptors for ethylene have now been identified (Bleecker, 1999) and signal transduction appears to involve protein kinases (Kieber *et al.,* 1993; Novikova *et al.,* 2000, Moshkov *et al.,* 2003a), monomeric GTP-binding proteins (Novikova *et al.,* 1997, 1999, Moshkov *et al.,* 2003b), transcription factors (Chao *et al.,* 1997) and other proteins of unknown function (Johnson and Ecker, 1998; Stepanova and Ecker, 2000). The receptors all contain Cu(I) in the binding domain (Rodriguez *et al.,* 1999). The downstream effects include altered gene transcription and protein synthesis.

3.3.1. Inhibitors of ethylene action

Several chemicals and environmental factors are known to inhibit ethylene action. These may either compete with ethylene for binding domains in the receptors or act by unknown mechanisms. Inhibitors of ethylene action have been used experimentally as an alternative to biosynthesis inhibitors, in conditions where ethylene is thought to affect growth or morphogenesis in tissue cultures.

Silver ions. Silver ions are known to overcome the action of ethylene on whole plants (Beyer, 1976a, b). Both silver thiosulphate ($Ag_2S_2O_3$) and silver nitrate ($AgNO_3$) are therefore effective in preventing ethylene action although the former is much more effective because it is readily translocated. They do not, as was once thought, appear to replace Cu(I) in the binding domain and in fact increase rates of ethylene biosynthesis in peas (Sanders *et al.,* 1991), but may act by inhibiting the copper transporter protein RAN1.

Carbon dioxide. High concentrations (5-10%) of carbon dioxide can antagonise some ethylene effects - for example inhibition of epicotyl or hypocotyl extension (Burg and Burg, 1967; Beyer, 1979). The effect is competitive *in vivo* (Burg and Burg, 1967) but does not appear to be a result of displacement of ethylene from its receptor (Sisler, 1982; Sanders *et al.,* 1991). On the other hand, CO_2 at atmospheric concentrations appears to be necessary for some responses to ethylene (Hall *et al.,* 1980).

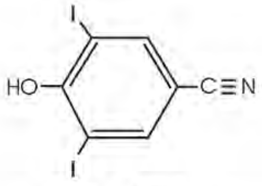
Chemical inhibitors. Although a wide range of chemicals [e.g. ioxynil (23) 3,5-diiodo-4-hydroxy-benzoic acid (DIHB) (24) and 5-methyl-7-chloro-4-ethoxycarbanylmethoxy-2,1,3-benzothia-diazole (benzothiadiazole TH6241; 25)] are known to block developmental effects of ethylene reputedly via effects on ethylene action, it is questionable whether this is so or whether the mechanisms' effects may be indirect and/or the result of modulation of ethylene biosynthesis. However, a number of hydrocarbons do specifically inhibit ethylene action by competing with the growth regulator for binding sites on its receptor. Such substances include 2,5 norbornadiene (26), cis-2-butene (27), cyclooctene (28) and methyl-cyclopropene (29) (Sisler, 1991; Sisler *et al.,* 1996). The latter is particularly effective, at concentrations as low as 10^{-9} M (Sisler and Serek, 1997).

Cytokinins. Cytokinins antagonise ethylene in many systems - for example leaf senescence - probably via early events in signal transduction (see e.g. Novikova *et al.,* 1999).

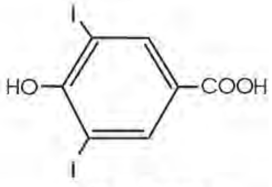
3.3.2. Ethylene-Releasing Chemicals

Several synthetic ethylene-releasing chemicals have been discovered. The one most commonly used in plant tissue culture experiments is ethephon (2-CEPA or 2-chloroethanephosphonic acid; 30). This compound is absorbed into plant tissues where it breaks down to release ethylene at cytoplasmic pH

levels. Cultures also usually produce more ethylene if the compound from which it is naturally derived, ACC, is added to the medium (Puschmann *et al.,* 1985). Adding methionine may also have the same effect (Druart, 1988).

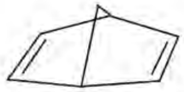


(23) ioxynil

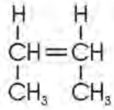


(24) DIHB

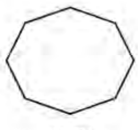
(25) TH 6241



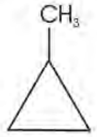
(26) 2,5-norbornadiene



(27) *cis*-2-butene



(28) cyclooctene



(29) 1-methylcyclopropene

$ClCH_2CH_2P(=O)(OH)_2$

(30) ethephon

3.4. ETHYLENE SOLUBILITY AND CHEMICAL ABSORPTION

Ethylene has a partition coefficient in water (concentration in aqueous phase ⁄concentration in gaseous phase) of 0.20 at 0ºC but only 0.10 at 20°C; so it is important to remember that the temperature at which a culture is maintained may have a marked effect on the cellular concentration and hence perhaps the developmental response. For experimental purposes, ethylene in the atmosphere of culture vessels can be be preferentially absorbed on a solution of potassium permanganate or mercuric perchlorate solution (Young *et al.,* 1951). The latter is the most efficient.

3.5. ETHYLENE PRODUCTION *IN VITRO*

Ethylene is produced during the culture of all kinds of plant cells, tissues and organs. The rate of biosynthesis is increased if cells are subjected to stress of some kind [e.g. high sodium chloride levels (Garcia and Einset, 1982, 1983) or toxic levels of ammonium ions]. Some chemicals added to culture media can also stimulate ethylene formation. Mannitol, which is commonly used in protoplast and other tissue cultures as an osmoticum, causes ethylene biosynthesis to be increased (Riov and Yang, 1982b); polyethylene glycol, which is sometimes used in protoplast fusion experiments, can also have the same effect. Potato protoplasts which had been treated with polyethylene glycol were much more likely to produce microcalli if 100 $mg2^{-1}$ $Ag_2S_2O_3$ was added to the medium (Perl *et al.,* 1988). Ethylene production is also greater during the senescence of cultured plant material: it reaches a maximum in suspension cultures at the start of the stationary phase when nutrients are limiting and most of the cells are senescing (LaRue and Gamborg, 1971). As noted, *Prunus avium* shoot cultures produced ethylene at a constant rate over 30 days (19 mlg^{-1} dry weight) (Righetti *et al.,* 1988) even when the CO_2 in the culture vessels reached 30% (Righetti *et al.,* 1990).

Suspension cultures of several different kinds of plants were found by Gamborg and LaRue (1968) and LaRue and Gamborg (1971) to produce ethylene at up to 1.22 µmole/hour/g dry weight of cells. The rate of evolution was greatest as the cells reached stationary phase, was the same in the light or the dark, and varied greatly between species (being especially low in cultures of monocotyledonous plants). Marked differences in the rate of ethylene production in different experiments were attributed to the differing physiological state of the cells. The amount of ethylene produced by callus cultures is also dependent on genotype (Thomas and Murashige, 1979a).

In sunflower suspensions, there appears to be no functional relationship between growth and ethylene synthesis, ethylene being just a by-product of actively dividing cells (Sauerbrey *et al.,* 1987).

As with whole plants or parts of plants, auxins generally increase the production of ethylene by cell and callus cultures but results have not always been predictable. This is unsurprising since, as noted

above, auxins can stimulate transcription of a specific isogene(s) for the ethylene biosynthetic enzyme ACS. Some at least of the effects of auxins on cultures are certainly mediated, at least in part, by ethylene. Production of ethylene by suspension cultures is dependent on the level of 2,4-D in the medium, but is independent of the effect of 2,4-D on growth (Mackenzie and Street, 1970; Sauerbrey *et al.,* 1987). Similarly, addition of 2,4-D to suspensions of *Ruta graveolens* promoted ethylene formation, but did not do so in cell cultures of *Rosa*, where only NAA and *p*CPA were stimulatory (Gamborg and LaRue, 1971). IAA increased ethylene production by suspension cultures of pear (Puschmann *et al.,* 1985).

Wulster and Sacalis (1980) observed that ethylene production from callus cultures of *Rosa hybrida* was increased by 1-3 mg/l of the auxins NAA or IBA, but was little affected by 5-15 mg/l of three cytokinins except when they were used in conjunction with auxin. Then the rate of ethylene production could be markedly increased. The concentration of cytokinin at which synergism occurred varied according to whether BAP, 2-iP or zeatin riboside was used. Lau and Yang (1973), who found a similar synergism in mung bean, discovered that ethylene evolution was closely related to the level of free IAA in the tissue. Kinetin promoted ethylene evolution because it prevented added IAA being converted rapidly into indoleacetylaspartic acid. In this conjugated form, the auxin had no effect on ethylene production. The amount of ethylene evolved by *Dahlia* callus was proportional to the concentration of NAA added to the medium (Gavinlertvatana *et al.,* 1982).

The addition of galactose to a medium has been found to increase ethylene levels within tissues (Colclasure and Yopp, 1976).

Ethylene production by habituated cultures is in general lower than in the normal hormone-dependent equivalents (see below).

3.5.1. Accumulation in culture vessels

As ethylene is produced from all kinds of plant cultures, including callus and suspensions, it accumulates in the gas phase within sealed culture vessels. The concentration in the free air space is found to vary according to the type of tissue being grown, the weight of the tissue, the volume of the culture vessel, the manner in which it is sealed, and the culture conditions.

The physiological effects of an ethylene atmosphere within vessels vary according to the concentration of the gas. The desirable and undesirable physiological effects of the concentrations with which cells or tissues are sometimes presented (particularly in containers that are tightly closed), are discussed below.

If the suspension cultures of LaRue and Gamborg (1971) produced ethylene at the same rate in tightly closed flasks as they did in foam-stoppered vessels, the air space in the flasks after 6 days of culture would have contained from only a trace (*Triticum monococcum*) to ca. 7.4 p.p.m ethylene (rose). Huxter *et al.,* (1981) measured 0.3-1.8 ppm ethylene in foam-stoppered flasks containing tobacco callus; Mackenzie and Street (1970) - 10 ppm in the air space above their sycamore suspensions; De Proft *et al.,* (1985) - up to 2-3 ppm above tightly stoppered *Magnolia* shoot cultures after 9 weeks.

The retention of ethylene depends of the nature of the closures used on culture vessels and on the tightness of the seal. Loosely covered flasks, such as those capped with aluminium foil, have been found to lose 50% of their ethylene in 2 hours, the rate of loss thereafter being constant (Gavinlertvatana *et al.,* 1982).

Ethylene can accumulate in flasks in significant quantities if their necks are momentarily flamed with an alcohol or natural gas burner during transfers (Beasley and Eaks, 1979). It should be noted however that the effects of closures do not necessarily reflect changes in gaseous components such as CO_2 and ethylene. Thus, Santamaria *et al.*, (2000), using *Delphinium* plantlets, concluded that improved stomatal performance, growth and survival of the plantlets in ventilated vessels was more likely due to increased flow of water rather than an improvement in the gaseous environment.

3.6. ETHYLENE EFFECTS ON CULTURES

3.6.1. Explant Isolation

Ethylene production by plant tissues is temporarily increased when they are wounded, and so is significant during explant isolation. Less ethylene was produced during the isolation of potato leaf protoplasts if shoots, from which the leaves were taken, had been previously cultured with silver thiosulphate; the quantity of ethylene decreased still further if acetylsalicylic acid was added during the maceration process (Perl *et al.,* 1988). The effects of wounding on growth and morphogenesis are discussed further below.

3.6.2. Suspension cultures

In the cell cultures of LaRue and Gamborg (1971) described above, exogenous ethylene could not replace an auxin, which was required to induce cell division. Ethylene naturally accumulated within flasks appeared to have little effect, and there was only a 20-30% reduction in cell yield of *Ruta* and *Rosa* suspensions when relatively large amounts of ethylene were artificially added. The addition of ethephon did not stimulate cell division or growth of sycamore cells (Mackenzie and Street, 1970).

3.6.3. Callus initiation and growth

Growth stimulation. It might be expected that the accumulation of ethylene in culture vessels would induce the formation of callus, because in whole plants, undifferentiated callus-like growths (intumescences) have been seen to grow from the stems (Wallace, 1928; Evanari, 1961) or roots (Wilson, 1966) of intact plants, which have been kept for prolonged periods in low ethylene concentrations.

Ethylene does seem to stimulate callus growth of some plants *in vitro*. For example, on a hormone-free medium, *Ginkgo biloba* embryos germinated when placed on medium within tubes sealed with cotton plugs, but gave rise to callus if the tubes were covered with Parafilm (Webb *et al.,* 1986). Furthermore, although it has been thought that the increase in ethylene evolution caused by 2,4-D is independent of the effect of the auxin on growth (MacKenzie and Street, 1970), a close correlation has been found between the rate of ethylene production and the growth rate of tobacco (Huxter *et al.,* 1979; Bridgen and Lineberger, 1981), *Dahlia* (Gavinlertvatana *et al.,* 1982) and tomato (Mukund *et al.,* 1988) calluses. Several chemicals known to inhibit ethylene biosynthesis also reduced growth and ethylene evolution in exact proportion.

Growth inhibition. Contrary indications have also often been obtained: Chalutz and DeVay (1969) showed that ethylene inhibited the formation of callus and suberised cork cells on the injured surfaces of sweet potato roots and seemed to have little effect on the subsequent division of undifferentiated cells. Similarly, Zobel and Roberts (1978) found that an ethylene concentration above 0.1 ppm. prevented the initiation of callus and tracheids from *Lactuca* pith explants and deduced that this was because the gas prevented the cells becoming determined or differentiated. Once the cells became programmed to divide and grow as callus, ethylene had no further effect.

In rice, callus necrosis was most likely to occur in cultivars which produced ethylene at a high rate. Callus growth of these plants was more strongly inhibited by a controlled gas mixture high in ethylene than was that of necrosis-tolerant cultivars (Adkins *et al.,* 1990).

Songstad *et al.,* (1991) showed that the ethylene action inhibitor $AgNO_3$ increased type II callus production from immature embryos of maize.

Other physiological effects. A promotory effect of ethylene on xylogenesis in soybean callus and *Lactuca* pith explants has been reported (Miller and Roberts, 1982; Miller *et al.,* 1985). Chlorophyll synthesis and chloroplast development *in vitro* is depressed by ethylene (Dalton and Street, 1976).

3.6.4. Morphogenesis

The role of ethylene in morphogenesis is by no means clear, although its action in inhibiting polar auxin movement (see Chapter 5) may be partly responsible for the observed effects. Besides influencing the determination of cells that will give rise to callus (see above), the gas can influence organ formation *in vitro*. From available results, it appears that there is often a critical concentration at which morphogenesis is stimulated; concentrations below and above this being respectively ineffective or inhibitory.

Determination. Ethylene appeared to inhibit the capacity of *Helianthus annuus* callus to produce adventitious shoots. The determination was influenced by treatments to the seedlings from which explants were derived. Hypocotyls of dark-grown seedlings of *Helianthus annuus* produced less adventitious shoots than those grown in the light. This seemed to be correlated with an effect of light on the sensitivity of the tissues to ethylene, because treating 3-day-old dark-grown seedlings with 10 μM AVG, inhibited ethylene production for 7 days; providing 4 days elapsed before cultures were initiated, callus from the hypocotyls of these seedlings produced the same number of adventitious shoots as light-grown callus (Robinson and Adams, 1987).

Adventitious shoot formation. Ringe (1972) reported that direct shoot formation on *Begonia* x *richmondensis* stem segments was inhibited by 2-20 mg/l ethephon and completely prevented by 200 mg/l (although these same concentrations promoted callus proliferation). Adding ethephon to media supporting callus growth of wild or cultivated carrot has been reported to inhibit root and shoot formation (Wochok

and Wetherell, 1971). However, Gonzalez *et al.,* (1997) showed that AVG inhibited shoot induction and elongation in nodal segments of *Populus tremula*. Ethephon and ACC on the other hand promoted shoot induction. Chraibi *et al.,* (1991) showed that $CoCl_2$ and $AgNO_3$ stimulated shoot formation from cotyledons of *Helianthus annuus*. Similar effects of $AgNO_3$ on shoot formation from cotyledons of *Cucumis melo* were demonstrated by Roustan *et al.,* (1992).

Thorpe and co-workers (Thorpe *et al.,* 1981; Huxter *et al.,* 1981) concluded that ethylene inhibited shoot formation during the first 5 days of the initiation period, but thereafter it speeded up the formation of primordia. Callus of wheat and *Nicotiana plumbaginifolia* cultured on Tran Thanh Van (1973) medium with 1 mg/l 2,4-D, formed adventitious shoots when the auxin was omitted. Shoot formation was inhibited if ethylene (70 mM ethephon) was present from the beginning of the culture period and was stimulated (and adventitious root formation simultaneously reduced) if 5-50 mg/l silver nitrate was present thoughout the culture period (Purnhauser *et al.,* 1987). Ethylene inhibitors have been found to have a similar effect in other species too. For example, adventitious shoot formation on cotyledons or hypocotyls of *Brassica* spp. was enhanced by adding AVG (1-10 μM) or $AgNO_3$ (5-30 μM) to MS medium containing auxin and cytokinin (Chi *et al.,* 1990).

Ethylene in culture vessels. The ethylene and carbon dioxide, which built up in closed culture flasks during the first 15 days of culture, promoted the formation of shoot buds from *Pinus radiata* cotyledons. The additional ethylene was especially promotory during the first few days of culture but, if flasks were stoppered with foam plugs for 10-15 days, there was only a low frequency of shoot bud formation. Excessive concentrations of ethylene, which accumulated after 15 days (e.g. 25 ml/l at 25 days) caused a slight dedifferentiation of buds (Kumar *et al.,* 1986, 1987).

Formation of apogamous buds in the fern *Pteridium aquilinum* was also stimulated most effectively by a certain concentration of ethylene. Gametophytic colonies exposed to air produced only 4 buds per gram of tissue, whereas those exposed to 0.7 ppm. ethylene gave rise to 32. Less apogamous buds were formed by colonies in 2.5 and 14.5 ppm ethylene (Elmore and Whittier, 1973).

The number of adventitious buds formed on bulb scale explants of lily was increased if 1-10 ppm ethylene was applied in the gas phase above the cultures during the first 3-7 days of culture (Van Aartrijk *et al.,* 1985). A similar result was obtained by Taeb and Alderson (1987) with sections of the immature floral stems of tulips. Adding 1-10 μM ACC or 0.1-100 mM ethephon did not have the same promotory effect.

Bulb formation. The formation of bulbs on tulip shoots was enhanced by increased endogenous ethylene, achieved by adding 0.1-10 mg/l ACC to the medium, or by growing cultures under lights with a low red/far red ratio (Alderson and Taeb, 1990).

Root formation. Ethylene has been reported to have both promotory and inhibitory effects on rooting *in vitro*. Coleman *et al.,* (1980) showed that in conditions where 5 mM IAA promoted the formation of adventitious roots on tomato leaf discs, the simultaneous application of ethylene, or ethephon, was inhibitory. Furthermore, although natural ethylene production was stimulated by adding auxin to the medium, lowering its level in the culture flasks by aeration, absorbing it with mercuric perchlorate, or leaf disc pretreatment with $AgNO_3$ (1.7-17.0 mg/l for 30 min), doubled the number of roots formed. Although Coleman *et al.,* (1980) concluded that ethylene was not a rooting hormone per se, others have reported contrary indications. Fabijan *et al.,* (1981) suggested that although the initiation of roots from *Helianthus* hypocotyls was blocked by endogenous ethylene on the first day after excision, thereafter the gas was promotory.

Thin cell layers of *Nicotiana tabacum* were found to produce 100 times more ethylene on a root-inducing medium (containing 10 mM IBA and 0.1 mM kinetin) than tissues cultured on other media. The appearance of roots was associated with a further increase in ethylene synthesis, which was maximal at 30ºC and only trivial at 15 or 40ºC, suggesting an enzyme-mediated process. If silver ions were added to the medium, root formation was blocked and adventitious shoot buds were formed (Le Guyader, 1987).

Providing oxygen was present, the rooting of chrysanthemum cuttings in an experimental mist system was slightly enhanced by adding 10 ppm ethylene in the gas phase (Soffer and Burger, 1988), and extensive wounding (likely to increase endogenous ethylene evolution) also increased rooting of mung bean cuttings except when they were treated with 10 μM AVG (Robbins *et al.,* 1981). Gonzalez *et al.,* (1997) showed that AVG inhibited root formation in nodal segments of *Populus tremula*

whereas ethylene was promotory. On the other hand, Khalafalla and Hattori (2000) showed that $AgNO_3$, cobalt chloride and acetylsalicyclic acid enhanced root formation on *Vicia faba* shoots regenerated on a medium containing thidiazuron. ACC inhibited root formation.

Embryogenesis. Size of culture vessel was found to be most important to obtain optimum rates of embryogenesis from tobacco anthers (Dunwell, 1979), suggesting that a volatile substance was stimulatory at a critical concentration. In embryogenic 'Shamouti' orange cultures, low concentrations of ethephon (0.01-1.0 mg/l) stimulated embryo formation, but levels above these were inhibitory (Kochba *et al.,* 1978), and excessive levels of ethylene accumulated within closed flasks, have been found to prevent embryogenesis (Spiegel-Roy and Kochba, 1980).

Ishizaki *et al.,* (2000) found that at low concentration (10^{-9} M) ethephon more than doubled the frequency of formation of embryogenic callus from explants of spinach, a process inhibited by $AgNO_3$. Conversely, ethephon completely inhibited embryo development whereas $AgNO_3$ markedly increased the number of embryos. Hatanaka *et al.,* (1995) showed that removal of ethylene from culture flasks reduced the number of somatic embryos produced from leaf disks of *Coffea canephora* whereas $AgNO_3$ and cobalt inhibited embryo formation - the latter effect being reversed by ethephon.

Other evidence also suggests that ethylene, normally produced by tissues, is inhibitory to embryogenesis. The addition of 1-3 mg/l $AgNO_3$ to anther cultures of many varieties of *Brassica oleracea gemmifera*, increased the production of somatic embryos, and allowed embryos to be obtained from experiments and varieties where there would normally have been very few (Biddington *et al.,* 1988). Similarly, by placing 50-200 μM $AgNO_3$ in the medium on which maize embryogenic callus was cultured for 21 days (before it was placed on a regeneration medium), the number of plants obtained per unit weight of callus was increased. Acetyl salicylic acid also improved regeneration, but was less effective than $AgNO_3$ (Duncan and Widholm, 1987).

Al-Khayri and Al-Bahrany (2001) showed that $AgNO_3$ increased embryogenic callus weight from date palm in the absence of cytokinin but not in its presence. Similar effects were noted upon the numbers of embryos formed, although here the number was reduced by an increase in $AgNO_3$ concentration. Fernandez *et al.,* (1999) found that $AgNO_3$ enhances the induction of direct somatic embryogenesis from the scutellum of immature embryos of *Triticum durum*.

That ethylene is inhibitory to some early step in embryo initiation was suggested by the results of Duncan and Widholm (1988). Callus of *Zea mays* derived from immature embryos cultured with 15 mM dicamba (medium D) which produced somatic embryos when moved to a regeneration medium without the auxin (medium H), tended to regenerate many more plants from somatic embryos if 200 μM $AgNO_3$ had been added to the D medium. Tisserat and Murashige (1977c) found that embryogenesis of carrot and *Citrus media* was inhibited if ethephon was added to the medium. However the inhibitors of ethylene biosynthesis, AVG, amino-oxyacetic acid, 2,4-dinitrophenol and salicylic acid, prevented embryogenesis in *Medicago sativa*. Meijer and Brown (1988) suggested that these compounds were possibly blocking a biosynthetic pathway, other than ethylene formation, of crucial importance to embryogenesis. In the same connection Taylor *et al.,* (1994) observed that while $AgNO_3$ increased the yield of protoplasts and reduced shoot regeneration from sugarcane heterogeneous cell suspension cultures, application of either AVG or ACC had no effect on protoplast yield – suggesting that, at least as far as this process is concerned the role of $AgNO_3$ is probably not via an effect on ethylene action. Some of these apparently contradictory results may be due to differences between species, but, equally, may be due to the concentrations of ethylene used. Thus, Chen and Chang (2003) using *Oncidium* leaf cultures, observed that very low concentrations of ACC (5 and 10 mμM) retarded direct embryo formation from cut ends, but higher concentrations (20 and 50 mμM) accelerated the process.

Shoot growth and axillary bud break. Ethylene in the external atmosphere is generally inhibitory to cell growth in the meristems of seedlings (Fidler, 1960), whole plant shoots (Heck and Pires, 1962a, b), or roots (Chadwick and Burg, 1967; Andreae *et al.,* 1968; Radin and Loomis, 1969). Monocotyledons such as cereals and grasses are less affected than dicotyledons. Many species are extremely sensitive: pea seedlings for instance make noticeably less growth in an atmosphere containing 0.05-0.1 ppm ethylene (Fidler, 1960). It might therefore be expected that concentrations of ethylene found within culture flasks would prevent the normal growth of

directly or indirectly regenerated shoots, or of axillary shoots proliferating in shoot cultures. In fact, growth inhibition seldom appears to take place, and there is some evidence that ethylene may often have a stimulatory role (see introductory section). Adding 50-100 mg/l of the ethylene precursor methionine to the medium together with 1 mg/l BAP increased the rate of propagation of several woody species through several subcultures (Druart, 1988). Although their quality was poor, more shoots were produced by rose shoot cultures grown in sealed vessels (Horn *et al.,* 1988a, b), and low rates of exogenous ethylene (an injection of which gave an intra-vessel concentration of 5 p.p.m. that declined to zero over 10 days) were found to increase the rate of rose shoot proliferation. Higher concentrations (20 and especially, 100 p.p.m.) were inhibitory (Kevers *et al.,* 1992).

An intriguing experiment of Walker *et al.,* (1988) also suggests that ethylene affects the growth of *Rhododendron* shoot cultures. Firstly, the fresh weight and the number of shoots were reduced if culture vessels were flushed with a mixture of nitrogen, oxygen and carbon dioxide. Secondly, when cultures were grown in divided vessels with a common atmosphere, the presence in the medium on one side of the partition, of normal growth regulators (4 mg/l IAA and 15 mg/l 2-iP), increased the weight of shoots obtained from the other side when the shoots there were grown without regulants, or with IAA and 2-iP at half the above rates.

Stolon formation. Ethylene appears to be somehow involved in the regulation of stolon formation. Stolons are axillary shoots with the capacity for considerable internode extension. If metal caps covering jars for potato cultures were screwed down too tightly, there was a tendency for stolon-like shoots with small leaves to be produced; but shoots were short and swollen and had only scale leaves if the closures had been further covered with PVC tape or Parafilm. These ethylene-induced effects were eliminated by placing an ethylene absorbent (a vial containing mercuric perchlorate solution) into sealed jars or by replacing the metal covers with polyurethane foam (Hussey and Stacey, 1981; Creissen and Karp, 1985). Shoot cultures of *Fragaria ananassa* in covered vessels produced runners if silver nitrate (20 mg/l) and gibberellic acid (20 mg/l) were added to Boxus (1974a) medium. Without silver nitrate, gibberellic acid only caused the elongation of normal shoots (Zatyko *et al.,* 1989).

Growth inhibition. Some cases of ethylene-induced inhibition of shoot growth have been reported. The growth of cultured *Kalanchoe blossfeldiana* shoots was inhibited by the ethylene which built up in sealed tubes. There were less nodes per plant and a higher proportion of yellow leaves (Horn *et al.,* 1988a). Torres *et al.,* (1981) have reported that shoot formation from *in vitro* cultures of *Begonia* x *hiemalis* seemed to be inhibited in sealed flasks, although little difference was observed with Easter lilies or *Exacum*. When carnation shoot cultures were grown in tightly sealed vessels, sufficient ethylene was produced to reduce shoot internode length and cause leaves to become yellowish in colour and more swollen than those of the controls (Melé *et al.,* 1982). Ethylene may normally be prevented from inhibiting the growth (rather than formation) of shoots in shoot cultures, through the presence of cytokinin in the growth medium.

Bud dormancy. Although the dormancy of buds is preserved in the presence of external ethylene, once the plant or plant organ is removed from the gas, dormancy is broken and shoot growth is often more rapid than it might otherwise have been [e.g. that of potato tuber sprouts (Elmer, 1936)]. The dormancy of axillary buds on cuttings of woody plants such as apple, plum, cherry and willow is also broken when shoots, which have been kept for some while in an ethylene atmosphere, have subsequently been removed (Vacha and Harvey, 1927). For micro-propagation purposes, cytokinins are used to promote the growth of axillary shoots, and so in this instance cytokinins and the previous presence of high ethylene levels have the same final effect.

Accumulation of metabolites. A number of workers have observed that ethylene may modulate the accumulation of several secondary metabolites in plant cell cultures. Thus, Guo and Ohta (1994) found that ethylene promoted and AVG reduced the amount of 6-methoxymellein produced by suspension-cultured cells of carrot. Mirjalili and Linden (1995) found that the presence of ethylene in suspension cultures of *Taxus cuspidata* enhanced taxol production; on the other hand in suspension cultures of *Taxus chinensis* and *Taxus yunnanensis* ethephon decreased paclitaxel production and inhibitors of ethylene biosynthesis and silver nitrate increased it (Zhang and Wu, 2003). Yahia *et al.,* (1998) showed that ethylene stimulated indole alkaloid accumulation in cell suspension cultures of *Catharanthus roseus*. Interestingly, cytokinin also enhanced accumulation and the responses to this hormone and ethylene were additive. On the other hand, Lee and Shuler (1991)

found that ethylene inhibited the production of ajmalicine production in the same type of cultures. Berglund and Ohlsson (1992) also showed that AVG increased and ethephon decreased the accumulation of cardenolide in tissue cultures of *Digitalis lanata*.

3.7. OTHER VOLATILES

Volatile substances apart from ethylene are produced and evolved from cultured plant tissues. Methane, ethane, acetaldehyde and ethanol have all been detected in the gas phase above tissue cultures (Thomas and Murashige, 1979a, b; Torres *et al.,* 1981), some of which are the products of inefficient respiration in conditions of oxygen deficiency: ethane is produced by cells, which have been wounded and have suffered membrane damage (Van Aartrijk *et al.,* 1985). Embryogenesis in carrot and date palm callus can be inhibited by ethanol evolved by cultures (Tisserat and Murashige, 1977b; Thomas and Murashige, 1979a, b). Acetaldehyde has been thought to inhibit aerobic respiration, thereby stopping cell division (Laties, 1962). Methyl jasmonate (see below) is also volatile.

4. OTHER MESSAGES AND MESSENGERS

Until relatively recently, auxins, gibberellins, cytokinins, abscisic acid and ethylene were considered to be the only 'classical' plant hormones or natural plant growth substances. It is now clear that this is an oversimplification and that a number of other substances should be considered in this category - for example brassinosteroids, jasmonate and systemin. Yet others - such as polyamines - cannot be considered as hormones, but they are undoubtedly plant growth regulators. On the whole these other substances have so far found little application in tissue culture, (other than polyamines) but this is likely to change.

4.1. POLYAMINES

All higher plants contain aliphatic amine compounds which are necessary for growth. The most common of these compounds are putrescine (a diamine) (see Fig. 7.4), spermidine (a triamine) (Fig. 7.4) and spermine (a tetraamine) (Fig. 7.4), although there are many others. In solution, at cytoplasmic pH levels, these polyamines act as polycations and complexing agents. Polyamines are synthesized by plant cells in culture (Smith *et al.,* 1977).

Polyamines have been found to act as growth stimulants and sometimes to enhance the action of plant growth substances. It has been suggested that they may function as secondary messengers for hormones within cells (Smith, 1985), but this hypothesis is questioned (Fobert and Webb, 1988) and no further evidence for it has since been advanced. However, they are undoubtedly important in metabolism and development in plants (see Chattopadhyay and Ghosh, 1998; Malmberg *et al.,* 1998) and there is strong evidence that they are involved in resistance to both biotic and abiotic stresses (for reviews see Bouchereau *et al.,* 1999, Walters, 2000) Some advantages have been shown to accrue from their addition to plant culture media.

4.1.1. Biosynthesis

The biosynthetic route to polyamines from the precursors arginine and ornithine has been determined (Fig. 7.4) although the pathways, which are operative may vary from one species to another, and according to whether morphogenesis, or growth, is taking place (Feirer *et al.,* 1984; Tiburcio *et al.,* 1987). Ornithine decarboxylase (ODC) is thought to be chiefly activated during cell division (Heimer *et al.*, 1979; Bagni *et al.*, 1983), arginine decarboxylase (ADC) being activated during cell elargement (Galston, 1983). Interestingly, *Arabidopsis thaliana*, used widely in molecular genetic studies, appears to lack ODC (Hanfrey *et al.,* 2001).

The biosynthesis of polyamines is influenced by the medium used for *in vitro* culture; that of putrescine has been shown to be much greater on ammonium-based nutrition than when nitrogen is supplied as nitrate ions (Smith and Wiltshire, 1975; Altman and Levin, 1993). This may be in response to the reduction of medium pH which is associated with uptake of NH_4^+, although it has been suggested that putrescine accumulation may result when ammonium competes for potassium at a site of metabolic importance (Le Rudulier and Goas, 1975).

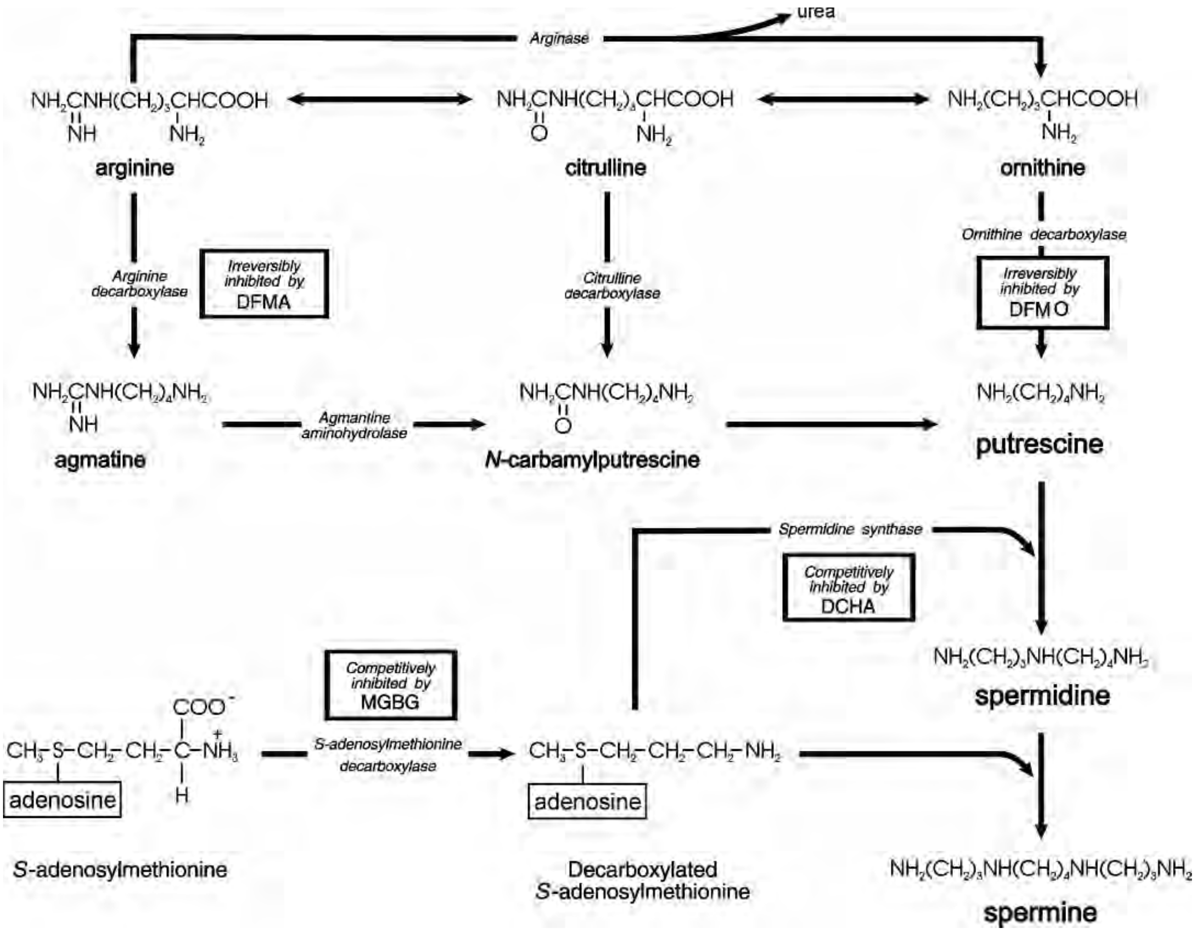


Fig. 7.4 Biosynthetic pathways to common polyamines.

4.1.2. Inhibitors of biosynthesis

Inhibitors of specific stages in the biosynthetic pathways illustrated in Fig. 7.4 have been discovered. They are:

- DFMA, DL-α-difluoromethylarginine (31);
- DFMO, α-difluoromethylornithine (32);
- MGBG, methylglyoxal-*bis*-(guanylhydrazone) (33).

The cyclohexylammonium ion is also inhibitory: it is commonly made available from one of two compounds, viz:

- DCHA, dicyclohexylammonium sulphate (34);
- CHAP, cyclohexylammonium phosphate (35).

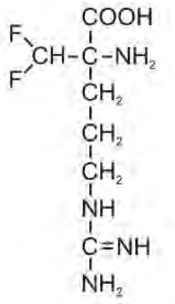
Very recently Bachmann *et al.,* (1998) have demonstrated that phaseolotoxin, a peptide produced by *Pseudomonas syringae phaseolicola*, is a potent inhibitor of ODC.

Responses obtained from addition of these inhibitors to plant culture media have been used to indicate the involvement of polyamines in growth and morphogenesis. DFMO has been said to be ineffective in certain plants (Galston, 1983; Flores and Galston, 1984). DFMO retards the growth of phytopathogenic fungi and it is claimed that plants treated with this inhibitor are protected from some kinds of fungal attack (Weinstein and Galston, 1988). Polyamine conjugates with hydroxylated cinnamic acids also occur in plants. They are thought to have regulatory properties and to be used by plants to inhibit the multiplication of viral pathogens.

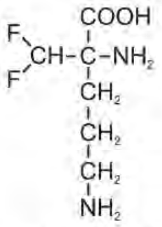
4.1.3. Physiological activity

At physiological pH, polyamines act as cations and bind strongly to nucleic acids and proteins, which

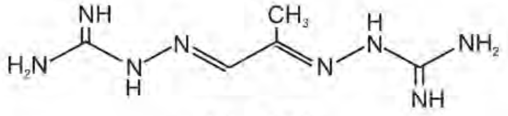
carry negatively charged groups. RNA and DNA are stabilized by their association with polyamines and in the bound form are more resistant to nuclease enzymes and thermal denaturation (Galston *et al.,* 1980). Free polyamines can compensate for ionic deficiencies or stress (Spector, 1989) within plants and can temporarily substitute for, and sometimes have the same physiological effect as, the cations K^+, Ca^{2+} and Mg^{2+}. Part of their function may be to act as buffers to minimize fluctuations in cellular pH (Slocum *et al.,* 1984). The concentration of putrescine is high in plants grown in conditions which favour the production of H^+ ions, such as acid stress, or where ammonium ions are provided as the major nitrogen source.



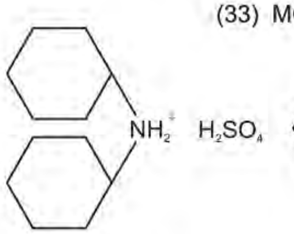
(31) DFMA



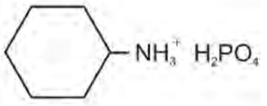
(32) DFMO



(33) MGBG



(34) DCHA



(35) CHAP

The modulation of growth caused by these compounds probably springs from their ability to bind to phospholipid groups and other anionic sites on membranes, cell wall polysaccharides and nucleic acids such as DNA, tRNA and mRNA. They are thought to modulate enzyme activities, enhance DNA replication and transcription and to be necessary to convert the structure of tRNA from an inactive to active form (Bagni and Biondi, 1987).

Levels of polyamine compounds tend to be highest in actively growing tissues and organs, such as root tips, buds (Kulpa *et al.,* 1985) and cells and tissues undergoing, or which have just undergone, morphogenesis (Tiburcio *et al.,* 1987; Wang and Faust, 1986; Martin-Tanguy *et al.,* 1988).

4.1.4. Interaction with growth regulators

Auxin activity. Exogenous polyamines have similar effects on plants to auxins, are directly involved in cell division (Bagni, 1966) and cell elongation (Bagni, 1986), and in certain circumstances can be used as a substitute for auxin treatment (Bagni *et al.,* 1978). Both auxins and polyamines are thought to act at cell membranes together with Ca^{2+} ions. The promotion of peroxidase secretion by auxins depends on the presence of calcium but polycations cause a rapid release of Ca^{2+} from the plasma membrane (Kevers *et al.,* 1985).

In *Sideratis* callus, 10 mM spermidine was able to annul the toxic effect of supra-optimal auxin or cytokinin levels (Sanchez-Graz and Segura, 1988). This may be related to the effects of polyamines on ethylene biosynthesis (see below).

Ethylene. Because polyamines and ethylene share a common precursor (*S*-adenosylmethionine, see Fig. 7.3) much attention has been devoted to their possible relationship. Polyamines can certainly inhibit ethylene biosynthesis, probably by inhibiting ACO (Apelbaum *et al.,* 1981); equally, ethylene can inhibit polyamine synthesis, by reducing the activity of ADC (Palavan *et al.,* 1984). Blocking ethylene production with the inhibitor AVG lends to increased spermidine production (Even-Chen *et al.,* 1982). There are also many reports that DFMO and DFMA treatment of plants leads to increased ethylene production. However, in some cases ethylene can increase putrescine levels (Lee and Chu, 1992). It should be noted that these latter findings were made in rice coleoptiles, where, in contrast to most other plants, ethylene promotes elongation, so this may be a special case. It is clear that a relationship between polyamines and ethylene does prevail *in planta*. For example Bisbis *et al.,* (2000a) showed that whereas fully habituated non-organogenic sugarbeet callus overproduces polyamines relative to its normal hormone-dependent counterpart, the reverse is true for ethylene production. In the same work, treatment of the habituated callus with both DFMA and DFMO reduced polyamine content and promoted ethylene production.

Gibberellic acid. In dwarf peas, gibberellic acid enhances the activity of ADC and ODC, with the result that polyamine levels in tissues are increased (Daie *et al.,* 1982; Galston, 1983; Galston *et al.,* 1983). The quantities of polyamines in genetic lines of peas differing in growth habit from very dwarf to tall, has also been found to be correlated with internode elongation and extractable gibberellin content. Inhibitors of polyamine synthesis also prevent internode elongation of dwarf lines by gibberellic acid (Smith *et al.,* 1985). However polyamines seem to affect only cell division, whereas gibberellin-induced growth involves both cell division and cell elongation. It was therefore concluded that polyamines are involved with only some aspects of these processes.

Cytokinins. Like gibberellins, cytokinins have also been found to increase the polyamine content of several different kinds of plant tissue (Suresh *et al.,* 1978; Cho, 1983; Palavan *et al.,* 1984), apparently because they increase the activity of arginine decarboxylase. Polyamines (and to a lesser extent arginine and lysine) delay leaf senescence in plants, and so have a similar physiological effect as cytokinins (Slocum *et al.,* 1984). It may also be significant that cytokinins commonly antagonise the effects of ethylene in accelerating leaf senescence and here again it is possible that the effects involve an interplay between ethylene and polyamines. Cadaverine, putrescine (1 mM), spermidine and spermine (0.1 mM), all inhibited the senescence of *Rosa* suspension cultures (Muhitch *et al.,* 1983). Polyamines are thought to delay senescence by stabilizing membranes (Altman, 1979; Srivasta and Smith, 1982), by interfering with the release or activity of hydrolytic ribonuclease and protease enzymes (Galston *et al.,* 1978), preventing lipid peroxidation, and protecting nucleic acids against nuclease degradation. Some synthetic chelating agents have similar effects (Shoemaker *et al.,* 1983).

Jasmonate. Lee *et al.,* (1996) showed that induction of chilling tolerance in rice led to increased levels of putrescine and spermidine and increases in the activities of ADC and SAMDC. The induction of tolerance was reduced by DFMA. Methyl jasmonate (see below) upregulated polyamine metabolism in thin layer explants of tobacco and this was associated with differences in growth and organogenesis (Biondi *et al.,* 2001).

4.1.5. Activity in tissue cultures

Cell division. Putrescine (50-100 μM) and ornithine (25 μM) have been found to stimulate cell division and callus colony formation from *Alnus* protoplasts (Huhtinen *et al.,* 1982); putrescine, spermidine and spermine improve the plating efficiency of sweet potato protoplasts through interaction with growth regulators (Eilers *et al.,* 1988).

Unconjugated polyamines do not appear to promote callus initiation, but do seem to improve the growth of callus when combined with sub-optimum levels of auxin and cytokinin. Perhaps this is because other growth regulators promote natural polyamine synthesis? If DCHA was added to the medium, callus growth from *Sideratis angustifolium* hypocotyls was decreased in the presence of auxins (Sanchez-Graz and Segura, 1988).

Spermidine (10 μM) or spermine (100 μM) were found to stimulate the growth of *Helianthus tuberosus* tuber explants and callus in a similar way to 0.05 μM IAA (Bagni, 1966; Bertossi *et al.,* 1965). *Nicotiana sylvestris* callus was stimulated by 90 μM putrescine (Oshmarina *et al.,* 1982), and *Sideritis* callus by spermidine (Sanchez-Graz and Segura, 1988). Hormone-induced dedifferentiation of root cultures of *Datura stramonium* was markedly inhibited by DFMA and the effect could be reversed by the addition of putrescine (Ford *et al.,* 1998).

The rapid growth of crown gall tumour tissue is thought to be associated with its high polyamine content (Kulpa *et al.,* 1985).

Polyamines conjugated with phenolic acids such as cinnamic, coumaric, caffeic and ferulic derivatives (see Chapter 5) are found in many plants (Smith, 1977). Martin *et al.,* (1985) found that putrescine could promote cell division if conjugated with a cinnamic acid. Out of several cinnamoyl derivatives tested, the best was caffeoyl putrescine, where 0.25 mM induced the maximum rate of cell division. Higher concentrations inhibited cell division, but were not toxic. When hydroxycinnamoylputrescine was added to a medium containing BAP, leaf discs of *N. tabacum* formed undifferentiated callus, instead of direct adventitious buds (Martin-Tanguy *et al.,* 1988).

Adventitious shoot formation. Polyamines may regulate adventitious shoot formation. Inhibitors of polyamine synthesis have been found to reduce the number of adventitious buds formed from chicory roots (Bagni and Biondi, 1987) and cotyledons of *Pinus radiata* (Biondi *et al.,* 1986). Shoot regeneration from apple leaf explants was enhanced

by adding 0.1-1 mM putrescine to a medium containing 0.5 mg/l NAA and 2 mg/l BAP (James *et al.,* 1988).

DMFO was ineffective in preventing adventitious bud formation in tobacco, although ODC appeared to be particularly involved in the production of polyamines required for subsequent growth of the primordia once they had been formed (Tiburcio *et al.,* 1987). In maize, however DMFA promoted indirect shoot formation: embryo-derived calluses of maize were cultured for three months on a modified MS medium containing 9 μM 2,4-D and 0.5 mM DMFA, and then moved the cultures to the same medium with 1 μM 2,4-D (and no inhibitor) (Tiburcio *et al.,* 1991). Many more calluses formed adventitious shoots than if the inhibitor had not been present. The number of plants obtained per callus was also four times greater and the plants developed more rapidly than the controls.

Adventitious roots. Inhibitors of polyamine synthesis have been found to prevent the formation of roots by thin cell layers excised from the floral stems of tobacco (Tiburcio *et al.,* 1989; Torrigiani *et al.,* 1990). Synthesis of endogenous polyamines increased prior to the formation of root primordia and during root growth on cuttings and hypocotyls of *Phaseolus* and *Vigna* treated with IBA (Friedman *et al.,* 1982, 1985; Jarvis *et al.,* 1983, 1985). Furthermore, in these plants, rooting was promoted by 50 mM spermine in the presence or absence of IBA, a result confirmed in *Phaseolus* by Fletcher *et al.,* (1988) and Kakkar and Rai (1987). Spermidine promoted adventitious root formation on root callus of *Sideratis*, but not on hypopcotyl callus (Sanchez-Graz and Segura, 1988). DFMO inhibits root formation from hypocotyls segments of *Euphorbia esula* and this can be partly overcome by treatment with IAA or 2,4D (Davis, 1997)

Light of 60 mmol m^{-2} sec^{-1} flux density is normally inhibitory to the rooting of micropropagated olive shoots, but was found not to be so if 160 mg/l putrescine was added to the rooting medium (Rugini *et al.,* 1988). The compound promoted earlier rooting and increased the proportion of cuttings forming roots.

MGBG totally blocked rhizogenesis but strongly promoted tuber formation in *Solanum tuberosum* (Pal Bais and Ravishankar, 2002).

Flowering. Evidence is accumulating that polyamines are associated with floral development (see review by Evans and Malmberg, 1989). In thin cell layers of tobacco, DFMA diminishes adventitious bud formation, particularly that of floral buds (Tiburcio *et al.,* 1987). Moreover, spermidine is able to induce the formation of flower buds (Kaur-Sawhney *et al.,* 1988), an event, which has been said to be associated with the attachment of the compound to a unique protein (Apelbaum *et al.,* 1988). On the other hand, in *Chrysanthemum marifolium* while flowering was inhibited by DFMO there was no effect of DFMA.

Promotion of embryogenesis. That polyamines are required for somatic embryo development was suggested by the discovery of Feirer *et al.*, (1984, 1985) that the number of somatic embryos formed from wild carrot cultures was severely reduced by 1 mM DMFA, but that embryogenesis could be restored if 0.1 mM putrescine, spermidine or spermine were added to the medium. Prevention of embryogenesis by DFMA is accompanied by an inhibition of ADC activity and a reduction in polyamine levels (Robie and Minocha, 1989). DMFO was more effective than DMFA at inhibiting direct embryogenesis on *Solanum melongena* cotyledons; the addition of putrescine restored the incidence of embryo formation to that of the controls (Fobert and Webb, 1988). Altman *et al.,* (1990) have observed that MGBG inhibited the growth of embryogenic clumps in suspension cultures of celery. DCHA and CHAP also have some inhibitory effect on carrot embryogenesis (Fienberg *et al.,* 1984; Khan and Minocha, 1991).

Hypocotyl callus of *Sideratis* initiated with NAA failed to produce somatic embryos when transferred to a medium without regulants, but embryogenesis did occur if the initial medium contained 27 μM NAA and 0.01 mM spermidine (Sanchez-Graz and Segura, 1988). This compound also promoted plantlet formation from embryogenic clumps of celery (Altman *et al.,* 1990).

It is becoming clear however that the absolute level of polyamines is not necessarily the critical factor. Thus Koetje *et al.,* (1993) showed that in rice, DFMA suppressed suspension culture growth and plant regeneration from callus. DFMO inhibited suspension culture growth only in the absence of 2,4-D and had no effect on plant regeneration. Also in rice, while spermidine had an inhibitory effect on plant regeneration in fresh cultures, it promoted this process in long-term cultures, which showed a reduction in regeneration potential (Bajaj and Rajam, 1995). Treatment with DFMA also restored regeneration potential in the long-term cultures (Bajaj and Rajam, 1996). These effects were attributed to

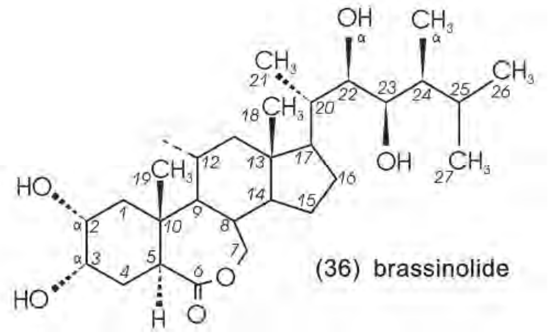
change in the spermidine/putrescine ratio. That this ratio is important was confirmed by Shoeb *et al.,* (2001) who showed that during rice embryogenesis, genotypes with good regenerative capacity had a putrescine/spermidine ratio of 2.3, those with moderate capacity a ratio of 3.8 while one genotype with a ratio of 10 showed no response at all.

Inhibition, or lack of involvement in embryogenesis. Bradley *et al.,* (1984, 1985) found that putrescine and arginine inhibited somatic embryo growth in wild carrot and could be used experimentally to synchronise embryo development. On a medium containing 40 mg/l arginine, cell suspensions or callus cultures of carrot produced somatic embryos asynchronously approximately 3 weeks after the auxin is withdrawn. But if 0.03 mM putrescine had been added to the induction medium, globular embryos were formed on the second medium (lacking the amine) but failed to develop further. Rapid and synchronous embryo growth of approximately half of these embryos occurred when they were moved to a medium without arginine.

No positive effect of added polyamines on either callus induction or somatic embryogenesis was observed from cultured nucelli of mangos (Litz, 1987) or with cotyledon explants of *Solanum melongena* (Fobert and Webb, 1988), although in the latter case DMFO (and to a lesser extent DMFA) caused there to be a reduction in embryogenesis and an increase in adventitious root formation. Polyamines did not significantly affect somatic embryogenesis in *Pinus taeda*.

5. STEROIDS

Until recently there was no definitive evidence that plant steroids were natural growth regulators - although application of both plant and animal steroids had been shown to elicit growth responses in plants (see below). There is now convincing evidence that brassinosteroids - of which the first was brassinolide (36) and of which group over forty related compounds are now known - are true hormones (Clouse and Sasse, 1998).



(36) brassinolide

The pathways of brassinolide biosynthesis are complex (Yokota, 1999) but involve the conversion of squalene to cycloartenol as a first step. A number of mutants of pea and *Arabidopsis* have defects in this biosynthetic pathway (see e.g. Fujioka *et al.,* 1997) and much work on the latter has been carried out using transformed and mutant cells of *Catharanthus roseus* (Fujioka *et al.,* 2000).

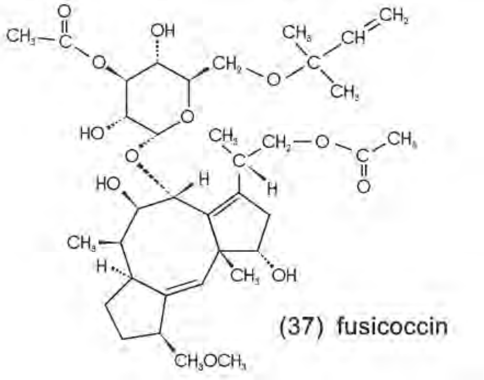
Little is known of the mode of action of brassinolide and other steroids but they appear to be involved in stem and root elongation and xylem differentiation (Iwasaki and Shibaoka, 1991). Some of these effects appear to be the result of alterations in the auxin response, but not all, and there is evidence for a role for light, either via effects on biosynthesis or signal transduction (Li *et al.,* 1996, Li and Chory, 1997, Brosa, 1999). The *BRI1* gene in *Arabidopsis* appears to code for a brassinolide receptor (He *et al.,* 2000).

In isolated *Zinnia* mesophyll cells brassinolide promted tracheary element differentiation (Iwasaki and Shibaoka, 1991), a process inhibited by the growth retardant uniconazole – presumably through an effect on brassinosteroid biosynthesis. Brassinolide has also been shown to accelerate early cell division in regenerating leaf protoplasts of *Petunia hybrida* (Oh and Clouse, 1998) and *Arabidopsis* (Hu *et al.,* 2000); it also increased growth in *Onosma paniculatum* cell cultures and enhanced shikonin formation (Yang *et al.,* 1999). It is beneficial in somatic embryogenesis in coconut palm (*Cocos nucifera*) (Azpeitia *et al.,* 2003) and in conifers and rice (Pullman *et al.,* 2003) and, in combination with BA, two analogues of brassinosteroids enhanced both callus formation an shoot regeneration from cotyledons of lettuce (Nunez *et al.,* 2004).

6. PHYTOTROPINS AND FUSICOCCIN

Highly specific receptor proteins for the auxin transport inhibitor naphthylphthalamic acid (NPA) [(8) in Chapter 5] have been detected in higher plants (see e.g. Rubery, 1990) suggesting that there exists an endogenous substance(s) which interacts with these receptors. This has not yet been identified with certainty but may be a flavonoid or flavonoid-like molecule such as quercitin (Table 5.2). It seems likely that the endogenous substances act in the same way as NPA, namely through the inhibition of auxin transport.

Similarly, the toxin fusicoccin (36) derived from the fungus *Fusicoccum amygdali* binds to a plant protein(s) with high affinity and specificity (see e.g. Aducci *et al.,* 1996), suggesting the presence of endogenous ligand(s) - for which there is some supporting evidence although the identity of these is unknown. Fusicoccin appears to mediate the effects of the protein(s) – which include promotion of growth and stomatal opening – via transmembrane ion pumps.



(37) fusicoccin

7. SYSTEMIN

The peptide systemin (38), which is produced in plants from its precursor prosystemin in response to wounding, appears to have a role in the development of resistance to pathogens via induction of proteinase inhibitors I and II. This effect is probably mediated via jasmonic acid. In suspension cultures of *Lycopersicon peruvianum* systemin induced transient alkalinisation of the culture medium and increased the activity of the ethylene biosynthetic enzyme ACS (Felix and Boller, 1995). The peptide is translocated - at least in tomato plants (Narvaezvasquez *et al.,* 1994; Leon *et al.,* 2001) - but the generality of its effects remain to be determined.

Ala-Val-Gln-Ser-Lys-Pro-Pro-Ser-Lys-Arg-Asp-Pro-Pro-Lys-Met-Gln-Thr-Asp

(38) systemin

8. SALICYLIC ACID

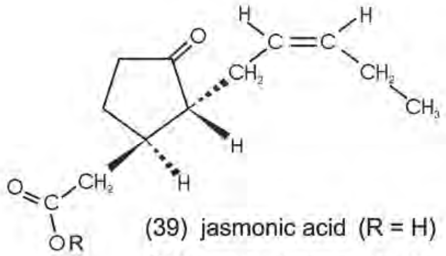
Salicylic acid (SA; Table 5.2) occurs widely in plants, being synthesized from *t*-cinnamic acid. It appears to have a role in systemic acquired resistance to pathogens and is able to induce various pathogen resistance proteins (Durner *et al.,* 1997). It also appears to be the substance, which controls thermogenesis in the floral spadix of some *Arum* spp. While SA is undoubtedly important in these cases, the generality of its effects and its possible function as a hormone remain to be proven.

9. NITRIC OXIDE

The important role of nitric acid (NO) as a messenger in animal systems is by now well established. However, there is increasing evidence that it has an important role in plant growth and development (Beligni and Lamattina, 2001a, b). Plants certainly have the necessary enzymic apparatus to produce the gas (Barroso *et al.,* 1999) and there have been reports of effects on plant disease resistance (Delledonne *et al.,* 1998), senescence (Leshem *et al.,* 1998) and stomatal closure (Mata and Lamattina, 2001). Very little has been done in relation to plant tissue and organ culture but the use of an NO donor - sodium nitroprusside - and an inhibitor of nitric oxide synthase, NG-monomethyl-L-arginine, in callus cultures from *Kalanchoe daigremontiana* and *Taxus brevifolia* indicates the involvement of NO in apoptosis (programmed cell death) (Pedroso *et al.,* 2000).

10. JASMONIC ACID

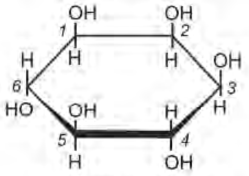
Jasmonic acid (JA) (39) and its methyl ester, methyl jasmonate (Me-JA) (40) have been shown to occur in many plants and synthesis is promoted by wounding via the action of systemin which activates a pathway in which linolenic acid is released from membranes (Ryan, 2000). Since JA induces the synthesis of a wide range of defence proteins, some of the effects of systemin are thought to be mediated by this compound (see also under the section on wounding below).



(39) jasmonic acid (R = H)
(40) methyl-jasmonate (R = CH_3)

JA has been used by a number of workers to modulate production of various secondary metabolites in tissue culture. Thus, Yukimune *et al.,* (1996) showed that Me-JA increased paclitaxel and related taxane production in cell cultures of *Taxus* spp. Reports of developmental effects in tissue culture are scanty but Camloh *et al.,* (1996, 1999) showed that JA stimulates the development of rhizoids and shoots in leaf cultures of *Platycerium bifurcatum* and can promote division of fern protoplasts. On the other hand Swiatek *et al.,* (2002) showed that jasmonate can freeze synchronized tobacco (*Nicotiana tabacum*) BY-2 cells in both the G1and G2 stages of the cell cycle.

12. *MYO*-INOSITOL



(41) *myo*-inositol

myo-Inositol (41) is an important component in plant metabolism (see e.g. Keller *et al.,* 1998) where it is involved in the synthesis of polyols, cell wall components and phosphoinositides. *myo*-Inositol often improves the growth of plant cultures but there is little evidence for a regulatory role – despite the fact that a derivative, *myo*-inositol-1,4,5-triphosphate, is a key factor in the phosphatidylinositol signalling system. Methylated derivatives such as *oninicitol* and *piritol* seem to have important properties as osmoprotectants (Ishitani *et al.,* 1996; Nelson *et al.,* 1998).

It should be noted however, that there is increasing evidence for interactions between simple sugars such as glucose, other than those concerned with the purely nutritional function of the carbohydrate (Gibson, 2004). For example, it has been shown that glucose enhances the degradation of a key transcriptional regulator in the ethylene signal transduction chain (Yanagisawa *et al*., 2003). This role of monosaccharides may have considerable implications for plant cell and tissue culture.

13. OLIGOSACCHARINS AND ELICITORS

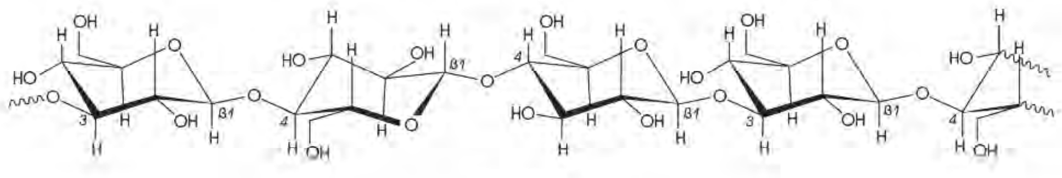
Oligosaccharins are complex sugars composed of one or more different monosaccharides. The three main types appear to be β-glucans (42), galacturonides (43) and xyloglucans (44). It has been suggested that these compounds naturally regulate cellular processes when they are released from cell walls by hydrolytic enzymes, which are activated by changes in pH, or by the presence of growth regulators such as auxins and cytokinins (Mohen *et al.,* 1985; Mutaftschiev *et al.,* 1987; Tran Thanh Van and Mutaftschiev, 1990). The degree of oligosaccharin polymerisation is critical for determining the biological activity of experimental samples prepared from plant cell walls using an

endopolygalacturonase enzyme or hydrolysis with a base (Tran Thanh Van and Mutaftschiev, 1990).

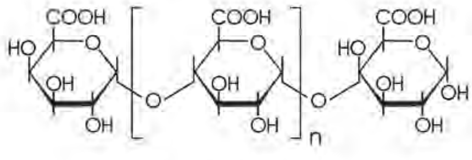
Synthesis of the enzymes (such as β-1,3-glucanase), which are responsible for the release of oligosaccharides, can be both induced and inhibited by auxin (York *et al.,* 1984; Mutaftshiev *et al.,* 1987) and mixtures of an auxin and a cytokinin (Mohen *et al.,* 1985). Other growth substances may also be involved (Albersheim *et al.,* 1988). Oligosaccharins have been shown to be capable of inducing cell enlargement and cell division in a similar fashion to IAA (York *et al.,* 1984; Mutaftshiev *et al.,* 1987) and to be capable of regulating morphogenesis (Tran Thanh Van *et al.,* 1985; Mutaftshiev *et al.,* 1987). Cell elongation caused by auxin can be either enhanced or inhibited by oligosaccharins. Because it is difficult to explain apical dominance satisfactorily by the translocation of IAA, Martin (1987) suggested that inhibition of lateral bud growth might be due to auxin at the apex inducing the action of β-1,3-glucanase. Oligosaccharins, which are consequently released from the walls of apical cells might then be transported to lateral buds, inhibiting their growth.

The effects of exogenous oligosaccharins are dose and pH dependent and can depend on the time at which they are introduced to the culture and the relative concentrations of auxin and cytokinin present in the medium (Mutaftschiev *et al.,* 1987; Tran Thanh Van and Mutaftschiev, 1990). Their mode of action is not determined, but they are thought to modulate the transcription of specfic genes (Guern, 1987).

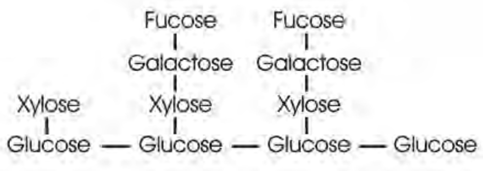
Interest in these substances by workers in plant cell and tissue culture seems to have diminished in the last decade, but this may be due to difficulties in obtaining sufficient of the compounds to carry out rigorous tests. Much more interest has been generated by elicitors - molecules of microbial origin that stimulate a number of defence responses in plants (Hahn, 1996). Such substances come in many guises - oligosaccharides, glycopeptides, peptides and proteins. Equally, substances such as alginates can often mimic the effects of elicitors (Akimoto *et al.,* 1999). Elicitors induce the formation of phytoalexins and other metabolic changes [for example increases in JA (Gundlach *et al.,* 1992)] in plant cells including those in suspension and tissue cultures. These properties have found some application by workers seeking to optimise the production of various secondary metabolites by tissue cultures. Thus, pterocarpen and isoflavone levels in chick pea cultures can be modulated by elicitors (Barz and Mackenbrock 1994) and large increases in sanguinarine production were observed in suspension cultures of *Esctischoltzia californica* after treatment with elicitors prepared from yeast extract, *Colletotrichum lindemuthianum* and *Verticillium dahliae* (Byun *et al.,* 1992). Treatment of late experimental phase hairy root cultures with pectinase led to a 150% increase in tabersonine (Rijhwani and Shanks, 1998) and elicitors from *Colletotrichum lindemuthianum* increased artemisin concentration in hairy root cultures of *Artemisia annua* (Wang *et al.,* 2001).



(42) ß-glucan



(43) An oligogalacturonide
In many bioassays, the polymer in which n = 10 is most active

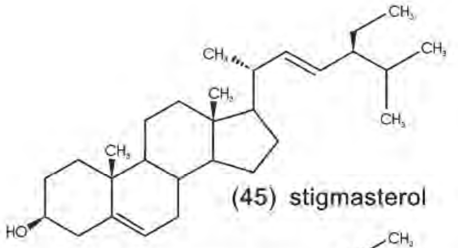


(44) A xyloglucan (XFFG)

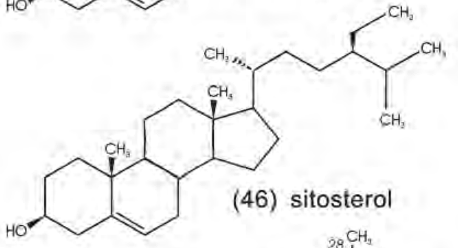
14. STEROLS

Plants synthesize a complex mixture of sterols; stigmasterol (45), sitosterol (46) and 24-methylcholesterol (47) are usually major components (Hartmann, 1998). They are important in the control of membrane fluidity but, perhaps equally importantly, they are involved in the synthesis of brassinosteroids (for a review see Piironen *et al.,* 2000). Indeed the phenotype of the dwarf pea mutant *lkb* is ascribed to a block in sterol metabolism leading to reduced levels of sitosterol and brassinosteroid (Nomura *et al.,* 1999). This may account for the effects of the sitosterol/24-methylcholesterol ratio on cell division in tobacco (Schaller *et al.,* 1998). It should also be noted that some of the effects of 'anti-gibberellins' may be due to effects on sterol metabolism (Rademacher, 2000). Given these roles it is unsurprising therefore that sterols may influence growth and development in culture. Thus, stigmasterol and vitamins D_2 (48) and D_3 (49) (which are required in mammals for proper bone structure), have been found to promote adventitious root formation in plants (Pythoud *et al.,* 1986; Talmon *et al.,* 1989). In shoot cultures of some cherry and apple cultivars, 1-10 ml/l of a water soluble formulation of vitamin D_2 enhanced the rate of propagation in early subcultures. After many subcultures with 10 ml/l, leaves became yellow (Druart, 1988).

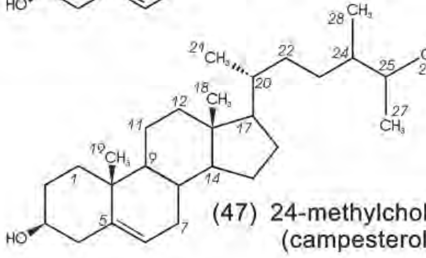
Rooting can be induced by relatively low concentrations of vitamin D_3 (e.g. 0.1 mg/l) but yet there is no damage to the plant from relatively high concentrations. Green cuttings of *Populus tremula* rooted best after being pre-dipped for 24 h at room temperature into a 0.25 µM (95.7 mg/l) solution which also contained 50 µM (10.2 mg/l) IBA (Pythoud *et al.,* 1986). The effect of vitamins D_2 and D_3 on stimulating root formation has been found to be additive or synergistic with that of auxins such as NAA (Moncousin and Gaspar, 1983), or IBA (Buchala and Schmid, 1979; Pythoud *et al.,* 1986); the number of roots formed depending on the age of the cuttings, the concentration of the compounds and the timing of the treatment. The rooting of cuttings produced by micropropagation has been said to be improved by adding a small amount of one of the D vitamins (e.g. 0.1-1 mg/l to an auxin preparation) (Pittet and Moncousin, 1982).



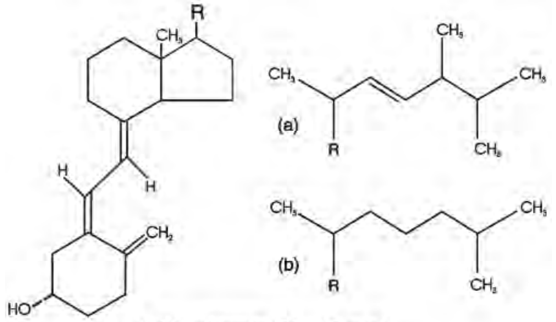
(45) stigmasterol



(46) sitosterol



(47) 24-methylcholesterol (campesterol)



(48) vitamin D2 (R = a)
(49) vitamin D3 (R = b)

15. UNUSUAL REGULANTS

15.1. COMPOUNDS WHICH CAN ARREST APICAL GROWTH

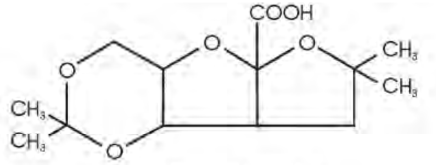
In shoot cultures, it is important to encourage the growth of lateral shoots, but genotypes vary in their natural degree of apical dominance: some produce a few long shoots while others have shorter shoots, but many laterals are formed. Perhaps genotypes with strong apical dominance may have a high natural

auxin content, or a more sensitive auxin receptor system (Newbury, 1986).

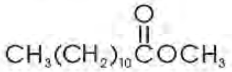
Although cytokinins are most frequently added to the medium to promote lateral branching, other techniques can be used to assist the process. Examples are reducing apical dominance by laying explants on their sides, removing the tips of shoots manually (Bressan *et al.,* 1982) and at each harvest subculturing the basal clump of tissue from which shoots have been removed. Some regulants can also help to remove apical dominance.

15.2. DIKEGULAC

Dikegulac (50) is a compound which, when sprayed over entire plants, is capable of arresting the growth of apical buds. Axillary buds are not inhibited and therefore grow out to produce a bushy plant. Dikegulac has been used routinely for this purpose on pot azaleas. Adding 500 or 1000 mg/l sodium dikegulac to the medium in which shoot cultures of sweet cherries were grown, caused an increased number of shoots to be developed from axillary buds (Snir, 1982).



(50) dikegulac



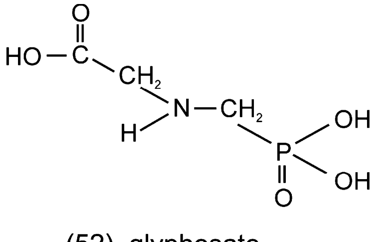
(51) methyl laurate
(methyl dodecanate)

15.3. METHYL LAURATE AND OPE

Methyl esters of fatty acids have been applied routinely to field-grown tobacco to prevent the growth of side shoots. Voyiatzi and Voyiatzis (1988) found that 100 mg/l methyl laurate (MELA) (51), added to MS medium containing 0.3 mg/l IAA and 3 mg/l BAP, increased the number of shoots produced by *Rosa hybrida* shoot cultures. The effect of the compound was initially similar to manual tipping but shoot number continued to be increased during further subcultures of the basal clump even though MELA was not then present. TIBA (3 mg/l) had an effect similar to manual tipping but only during the first passage.

When Jackson *et al.,* (1977) germinated seeds of *Colocasia esculenta* on Linsmaier and Skoog (1965) medium containing 5 mM of the surfactant octadecyl-polyethoxyethanol (OPE), they found that growth of the seedlings was restricted, but this was followed by the appearance of 5-10 shoots. Trial of this surfactant on shoot cultures is not reported.

15.4. GLYPHOSATE



(52) glyphosate

Glyphosate (52) is a general purpose post-emergence weedkiller which is translocated within both xylem and phloem. Winata and Harvey (1980) reported that sub-lethal doses, added to the medium used to induce callus from axillary buds of alfalfa, produced growth responses similar to those of more common growth regulators. In the presence of 0.17 mg/l glyphosate, the cultures produced rather more shoots than normal. If mother plants of some clones of lowbush blueberry had been sprayed with 250 or 500 mg/l glyphosate, single bud explants produced more shoots than usual during *in vitro* culture (Frett and Smagula, 1981). Scorza *et al.,* (1982, 1984) similarly found that when stem explants of cranberry were dipped in a 102.5 mg/l glyphosate solution for 30 seconds or a 321.2 mg/l solution for 5 seconds the development of both axillary and adventitious shoots was greater than usual when the explants were cultured afterwards on Anderson (1975) rhododendron medium containing 2-iP. Lower or higher levels of glyphosate were respectively ineffective or toxic. Stepwise selection carried out with increasing glyphosate concentrations to produce suspension cultures of alfalfa, soybean and tobacco leads to the production of glyphosate tolerant lines (100 fold more resistant than the original culture) (Widholm *et al.,* 2001).

15.5. ACTIVATED CHARCOAL

Finely-divided activated charcoal is often added advantageously to media at different stages of tissue cultures. Activated charcoal is not a growth regulator, but a discussion of its properties is conveniently included in this chapter on account of

its ability to modify medium composition, and thereby, in some circumstances, improve or regulate plant growth *in vitro*. The properties of activated charcoal vary according to the method by which it has been prepared, and not all brands are equivalent. A clear difference in the effectiveness of charcoal of different origins in promoting androgenesis was reported by Heberle-Bors (1980). As many of the beneficial effects of charcoal depend on its ability to absorb a wide range of compounds, results from even the most effective brands are liable to be unpredictable; inhibition of growth and morphogenesis are frequently observed.

Five kinds of advantageous uses of charcoal have been reported depending on the type of culture:

- to absorb compounds secreted from cultured tissues or present in agar that would otherwise inhibit growth;
- to prevent unwanted callus growth;
- to promote morphogenesis, particularly embryogenesis;
- to promote root formation (where some of the beneficial effect seems to be due to its ability to exclude light from the medium);
- to act as a reservoir during the production of secondary plant products in cultures;

A recent review on the effects and mode of action of activated charcoal may be found in Pan and van Staden (1998).

15.5.1. Mode of action of charcoal

Activated charcoal (AC) is prepared by the controlled carbonisation of wood in steam or air: it possesses strong absorptive properties and is used in chemistry to absorb both gases and dissolved solids. Activated charcoal added to tissue culture media was commonly thought to remove only growth inhibitory substances exuded by tissues or present in the ingredients of the medium; but it is now clear that promotory substances can also be absorbed and made unavailable.

Weatherhead *et al.,* (1979) pointed out that AC has the potential to absorb some inorganic ions. A sample of fresh unused AC analysed for these authors contained many inorganic impurities, of which iron, aluminium, nickel and magnesium might have had physiological significance, but there was no evidence that these metals were donated to culture media.

15.5.2. Absorption of growth inhibitors

Charcoal can assist in absorbing toxic substances, which may be present in media ingredients, produced as a result of autoclaving, or exuded by cultured tissues, particularly when they are first transferred to media. Fridborg *et al.,* (1978) showed that AC could adsorb some phenols commonly produced by wounded tissues, and this was later confirmed by Weatherhead *et al.,* (1979). Extracts of the charcoal that had been incubated in Bourgin and Nitsch (1967) agar medium showed that it had adsorbed 5-hydroxymethyl-furfural (HMF). This compound was thought to have originated from autoclaving sucrose in the medium under mildly acid conditions. Furthermore, HMF was shown to be somewhat inhibitory to embryogenesis in tobacco anther culture, unless activated charcoal was present (Weatherhead *et al.,* 1978).

Charcoal is sometimes used in bacteriological media to remove inhibitory fatty acids present in agar. That some agars can contain substances capable of hindering plant growth and morphogenesis was shown by Kohlenbach and Wernicke (1978). Somatic embryo formation from tobacco anthers on an agar medium was improved when agar was prewashed with water dialysed against activated charcoal. Better results were obtained by using highly purified Difco Noble agar rather than Difco Bacto agar. Tyagi *et al.,* (1980) also reported that effective concentrations of charcoal for promoting embryogenesis from *Datura* pollen, varied according to the type of agar employed. The atypical growth of *Pinus radiata* shoots on some brands of agar could be prevented if AC was added (Nairn, 1988).

15.5.3. Adsorption of organic compounds

Growth regulators. Fridborg and Eriksson (1975) suggested that activated charcoal removed growth regulators, particularly auxin, from the medium and evidence has indeed accumulated to show that this does occur. A protective coating of charcoal is able to protect seeds from herbicides in soil mixtures (Taylor and Warholic, 1987). In tissue culture media 0.1% (w/w) AC can effectively absorb 10 mM IAA (1.75 mg/l) and 10 mM IBA (2.03 mg/l) from liquid MS medium to the extent that these compounds can no longer be detected by high performance liquid chromatography (limit 0.05 mg/l) (Nissen and Sutter, 1988, 1990).

Examples of growth regulators in culture media, which have been rendered ineffective by AC, are shown in Table 7.1. Note that cytokinins and abscisic acid, as well as auxins, can be made unavailable. Jona and Vigliocco (1985) added charcoal (together with GA_3) to the medium on which *Prunus persica*

shoots were to be elongated, finding that it overcame the negative effect of the high rate of cytokinin previously used in a shoot proliferation medium.

AC can make comparatively high concentrations of growth regulators inaccessible to tissue cultures. Nissen and Sutter (1990) recommend that 10-100 times more auxin should be added to a medium if high concentrations of activated charcoal are also introduced. In cultures of palms, where up to 3% AC is added to prevent blackening, it is necessary to add 0.15-0.5 mM 2,4-D to induce embryogenesis (i.e. 5-20 times what would normally be required) (Tisserat *et al.,* 1979; 1981). To initiate embryogenic callus of oil palm (*Elaeis*), Paranjothy and Rohani (1982) had to use 10 times the level of auxin that would otherwise have been effective, and Nwankwo and Krikorian (1983), using 0.5 g/l AC, increased the concentration of NAA or 2,4-D from 5-10 mg/l to 10-70 mg/l. Activated charcoal is capable of trapping gases, and so may remove ethylene or other gases, released from cultured tissues (Ernst, 1974; Johansson *et al.,* 1982, Mensualisodi *et al.,* 1993), although in the latter case this absorption did not seem of itself to improve the growth of *Anemone* seedlings in culture since they produced more of the gas in the presence of active charcoal than in its absence.

Organic nutrients. AC may not only remove growth regulators from the medium, but also some organic nutrients. Weatherhead *et al.,* (1979) found that tobacco anther cultures were deprived of thiamine-HCl and nicotinic acid when 0.3% AC was added to Bourgin and Nitsch (1967) H medium. Biotin, folic acid and pyridoxine are also likely to be absorbed, but not *myo*-inositol

Excluding light from the medium. If sufficient AC is added to the medium it can exclude light sufficiently to promote physiological reactions, which occur in the dark. The absorptive properties of the substance are incidental, for lamp-black carbon or graphite can be used equally effectively.

Altering the pH of the medium. Several authors (Langowska, 1980; Rahbar and Chopra, 1982; Smith and Krikorian, 1990) have noticed that the presence of activated charcoal causes the pH of culture media to be higher than they would otherwise have been. Owen *et al.,* (1991) found that an increase of ca. 0.75 units occurred in the pH of MS medium in the presence of 0.5% charcoal; the pH change occurred partly during autoclaving and partly in the the subsequent first 14 days of medium storage. A similar increase in pH occurred with both hydrochloric acid washed and neutralised activated charcoal (Sigma Chemical Co.), but the final pH of the medium containing the former was ca. 6.2; that containing the netralised charcoal was ca. 6.7. It has been suggested that increase in pH could be due to the capacity of AC to absorb cations (Langowska, 1980).

Table 7.1. Examples of growth regulators absorbed by activated charcoal.

Growth regulator	AC % w/v	Reference
1mg/l NAA	1	Fridborg & Eriksson (1975b)
0.01-10 mg/1 NAA & IAA	1	Constantin *et al.,* (1976, 1977)
5mg/l BAP	0.3	Maene and Debergh (1985)
10 mg/1 BAP	0.5	Takayama & Misawa (1980)
2,4-D and 0.03 mg/1 IAA + 1 mg/1 kinetin	0.5	Scholl *et al.,* (1981)
0.002-2 mg/1 BAP & 2-iP + up to 300 mg/1 NAA	0.3	Weatherhead *et al.*, (1978)
1 mg/1 NAA + 10 mg/1 BAP	0.5	Steinitz & Yahel (1982)
ABA in cultured anthers	1&10	Johansson *et al.,* (1982)
2.64 mg/1 ABA	1	Johansson (1983)

As pH can influence culture growth and morphogenesis, it is probable that at least some of the effects of activated charcoal on plant cultures can be attributed to the changes it induces in the pH of the medium.

Other effects. It should be noted that sucrose hydrolysis is accelerated during autoclaving of media containing activated charcoal (1%) resulting in acidification and increase in osmolarity (Druart and Dewulf, 1993).

16. UNIDENTIFIED GROWTH FACTORS

16.1 THE WOUND RESPONSE

Wounding is a commonplace occurrence for plants in the field or the laboratory, whether herbivory, mechanical stress or attack by insects, infection by microorganisms or nematodes and so on. Equally, in plant manipulation for tissue and organ

culture, wounding is a feature of the techniques whether this be shear stresses in liquid culture, excision of plant parts and, more recently, in plant transformation - for example via particle guns or *Agrobacterium*.

It has become clear in the last decade that the response of plants to wounding - by whatever means - is extremely complex, but also tightly regulated. It has long been known that wounding leads to increased ethylene biosynthesis and that the effect is dependent on the expression of wound-specific genes for ACS. However, it is now clear that this is only part of the story. Mention has already been made of the induction of the mobile peptide systemin by wounding and of the fact that this in turn leads to increased synthesis of ethylene and jasmonate. This results in a galaxy of effects including changes in ion fluxes, induction of pathogen related proteins, proteinase inhibitors, antioxidant enzymes as well as other processes leading to increased lignification and cross-linking of cell wall components (e.g. Bradley *et al.,* 1992; McGurl *et al.,* 1994; Grantz *et al.,* 1995; Hiraga *et al.,* 2000). Aside from mechanical wounding itself other dimensions of this syndrome are provided by elicitors and oligosaccharins, which may induce similar and/or complementary effects. Indeed, it is notable that many other natural and unnatural stresses such as heat, cold, drought, salinity and synthetic growth regulators can also initiate metabolic events similar to those caused by wounding.

The key to understanding these phenomena probably lies in the fact that whatever the stimulus or signal - environmental or endogenous - the initial responses are mediated by systems similar to those found in animals, monomeric G-protein and protein kinase cascades, phospholipid signalling and so on. Moreover, these signalling pathways are not wholly independent but may interact in complex ways (for a review see Hall *et al.,* 2002).

Given the foregoing, it is unsurprising therefore that wounding tissue often leads to effects such as those seen after application of various substances such as hormones, elicitors and synthetic growth regulators which are described earlier in this chapter - for example effects on morphogenesis. However, we can only see through a glass darkly at this stage and the effect of wounding on any one system will depend on a multitude of factors, which we cannot presently define.

17. HABITUATION

The capacity of cells to change from a state of being dependent on the exogenous supply of a particular substance to one, in which they are wholly - or partly - sufficient, has been termed habituation: cells or tissues are said to be habituated to subtance X or substance Y. The habituated condition of cultured cells or tissues is usually self-perpetuating from one vegetative generation to another, but can be reversed. Clearly habituation can only occur to organic molecules, which can be synthesized by cells, and not to basic nutrients.

Although habituation usually describes a change in the requirement of cultured tissues, from needing an exogenous supply of a substance, to becoming self-sufficient, the definition could be extended to include all cells or tissues capable of self-sufficiency, whether or not the trait was developed in culture. Tissues habituated towards the production of a particular substance or type of substance are autotrophic for that compound, while non-habituated tissues are heterotrophic.

It has been found that *in vitro* habituation does not necessarily result in complete independence from exogenous growth factors. Meins (1974) showed that cells were capable of shifting into a variety of stable states of cytokinin-habituation and could also undergo changes into states of greater or lesser habituation (see below). Kevers *et al.,* (1996) showed that fully habituated organogenic sugarbeet calluses responded to auxins, both in terms of growth and ethylene production and that these treatments also resulted in changes in contents of cytokinin, polyamines and ajmalicine; a hypothesis supported by the work of Gaspar *et al.,* (1991) with sugarbeet calluses. Habituated tissues are often differentiated. Highly embryogenic callus [e.g. of *Citrus* (Button *et al.,* 1974; Spiegel-Roy and Kochba, 1980)] and shoot-forming calluses [e.g. of sugarbeet (de Greef and Jacobs, 1979; Van Geyt and Jacobs, 1985)] have been capable of growth and morphogenesis without the addition of exogenous growth regulants.

Habituation to growth regulators occurs commonly in plant tissue cultures, but plant cells may also become habituated to other organic compounds normally added to culture media to support cell growth. For example, Ikeda *et al.,* (1979) recorded the habituation of soybean cells to thiamine and its precursors and Savage *et al.,* (1979) the recovery of a

pantothenate autotroph from a cell line of *Datura innoxia* which previously required this vitamin for growth.

17.1. GROWTH FACTOR DEPENDENCE

Auxin- or cytokinin-habituations are found relatively frequently in tissue cultures and are generally very stable. Cells in this condition can remain independent of an exogenous growth factor supply for many generations, although typically the condition is eliminated when plants are regenerated. However, as noted above, habituation to a growth regulator does not imply insensitivity to it.

Kerbauy *et al.,* (1986) found that cytokinin-autotrophic callus could be isolated from tissue explanted directly onto media without cytokinin, and Jackson and Lyndon (1988) have showed that explants removed from the meristematic regions of tobacco are most likely to form cytokinin-autotrophic callus. Thus callus derived from internode pith of juvenile plants was autotrophic, but that derived from similar regions of a mature plant was not. Pith removed from a few internodes below that, which was habituated gave rise to callus which could become cytokinin-habituated when cultured at 35ºC. Cheng (1972) obtained auxin autotrophic callus from pith isolated near the apex of *Nicotiana glauca* x *N. langsdorfii* hybrids, but auxin-requiring (heterotrophic) callus from pith taken from below the 7th node.

17.2. INDUCTION OF HABITUATION

Meins (1974) showed that a cytokinin-habituated state can be induced in tobacco tissue by incubating explants at 35ºC without cytokinin. In subsequent incubation at 25ºC, no cytokinin was required for callus initiation or growth, unlike tissue incubated throughout at this temperature. However, cells habituated at 35ºC required exogenous cytokinin once again if incubated at 16ºC (Binns and Meins, 1979). Presumably endogenous synthesis of cytokinin had decreased at the lower temperature (or there was an accelerated rate of degradation). A reversal of cytokinin habituation by culturing tissue at 16ºC, instead of the usual 26ºC, was reported by Syono and Furuya (1971); a reversal of habituation is also commonly brought about by the regeneration of complete plants from cell clones autotrophic for cytokinin and/or auxin. Explants from the regenerated plants are unable to grow in culture without addition of the growth factor, which was produced endogenously by cells in the original habituated state. Bennici and Bruschi (1999) showed that the formation of callus habituated for both auxin and cytokinin could be induced with high frequency after a very short treatment of hypocotyls of *Nicotiana bigelovii* with 2,4D.

17.3. THE CAUSES AND EFFECTS OF HABITUATION

Meins (1974) and Meins and Lutz (1980) argued that reversible habituation results from an altered expression of the genes already present in the cell (their repression or derepression) and is epigenetic by nature, because the changes observed:

- are directed rather than random;
- are restricted by the genetic capacity of the cell;
- are potentially reversible;
- involve a large number of cell conversions per generation, and
- do not alter the totipotency of the altered cell.

Meins and Binns (1978) and Meins (1987) have proposed that cell division factors can induce the biosynthesis of naturally-occurring substances of the same kind, through a positive feedback mechanism (Fig. 7.5). It is not clear whether the rate of biosynthesis is accelerated or the rate of metabolism increased, and so one could predict either of the two scenarios shown in the figure.

- If according to the first case, kinetin were to cause the natural production of zeatin to be increased, and if this natural cytokinin were not metabolised sufficiently rapidly, the increase in overall cytokinin concentration would cause the level of zeatin biosynthesis to increase even further. Withdrawal of the kinetin trigger to zeatin production, would then leave the tissue in a cytokinin-autonomous state.
- The second hypothesis presumes that biosynthesis of zeatin and/or other natural cytokinins is normally balanced by the metabolism of growth substance which is not required for immediate use. This condition would be disturbed by the presence of an inhibitor of cytokinin metabolism which decreased the rate of biodegradation causing an increase in the level of free natural cytokinin. An elevated level of free growth substance must then be supposed to lead to an autocatalytic increase in biosynthesis.

Case 1 in Fig. 7.5 is supported by the discovery that kinetin can induce the increased accumulation of zeatin riboside in callus tissue (Hansen, reported in Meins, 1987). A similar condition is occasionally noted in shoot cultures of some species, where high endogenous levels of cytokinins are found after several subcultures (Boulay and Franclet, 1986; McCulloch, 1988). That case 2 can also occur, is shown by the findings that thidiazuron can inhibit the

cytokinin oxidase enzyme (see Chapter 4), and can induce cytokinin autonomous growth, for example in some *Phaseolus lunatus* genotypes (Mok *et al.,* 1982). However, this is certainly not the whole story. Thus, Lambe *et al.,* (1997) have presented evidence that there are marked changes in DNA methylation during long-term callus culture and suggest that auxin has a role in this process (and see also below under 'Effects of habituation').

17.4. GENETIC CONTROL

Although habituation of cultured tissues is usually reversible, it is now known that heterotrophic cells can also become permanently autotrophic as a result of somatic mutation in the genes governing the production of endogenous growth factors. Evidence that this might be the case was discovered by several workers; callus capable of growing on media without the addition of growth substances was, for example, obtained by exposing cultures to gamma radiation (Pandey and Sabharwal, 1979); the ability of some genotypes of *Phaseolus lunatus* to grow *in vitro* without the presence of cytokinin appeared to be under genetic control (Mok and Mok, 1979). Delbreil and Jullien (1994) using different genotypes of *Asparagus officinalis* demonstrated the random occurrence of highly embryogenic tissue, which could be continuously subcultured on hormone-free medium. Genetic analysis demonstrated that this was due to a dominant monogenic mutation.

Callus, re-initiated from plants derived from habituated sugarbeet callus, was again found to be habituated (Coumans *et al.,* 1982), as were plants regenerated from auxin- habituated callus of *Nicotiana bigelovii* var. *quadrivalvis* (Bennici, 1983).

Examples of stable and sexually transmissible 'habituations' are provided by Meins *et al.,* (1983) and Meins and Foster (1986).

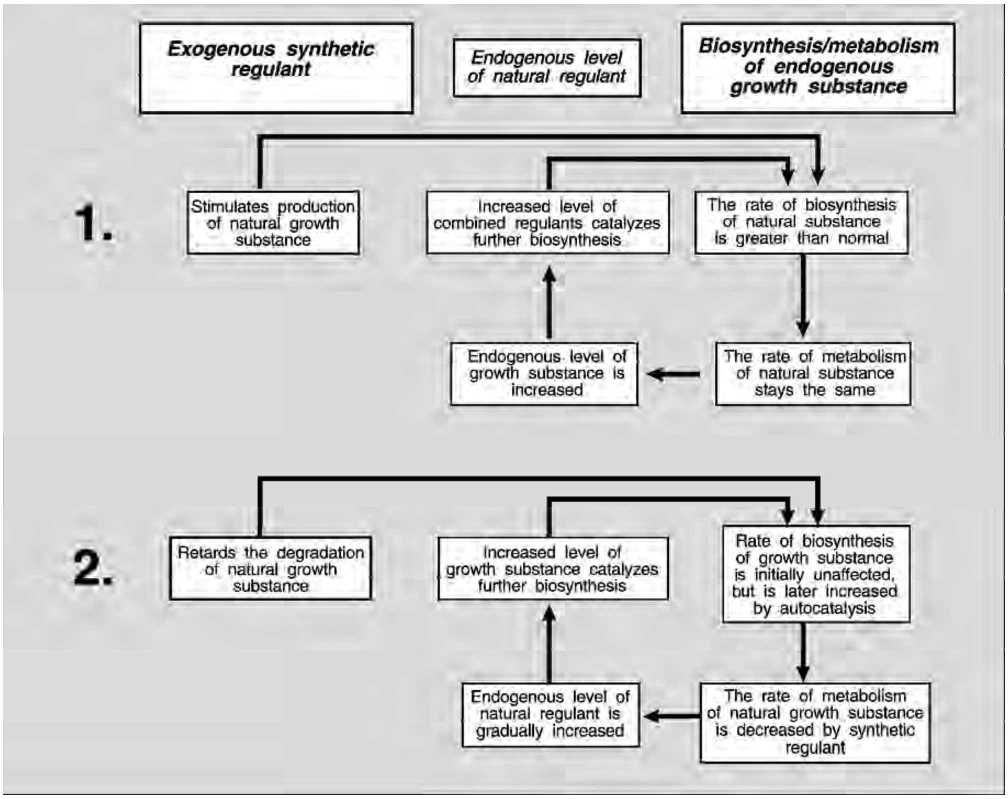


Fig. 7.5 Two possible explanations of growth regulator habituation in plant cultures.

17.5. EFFECTS OF HABITUATION

Habituated cultures show many differences in metabolism to normal cultures. This can take the form of changes not only in the growth regulators to which the callus has become habituated but also of others. Thus, Bisbis *et al.,* (2000a) showed that fully

habituated non-organogenic sugarbeet callus produces less ethylene and more polyamines than its hormone-dependent counterpart. There are also marked differences between habituated and non-habituated lines in terms of general metabolism. Thus, both Poder *et al.,* (1998) and Bisbis *et al.,* (2000b) showed that cyanide-resistant respiration was much higher in habituated than in normal callus (both organogenic and non-organogenic). Likewise Hagege *et al.,* (1992) demonstrated that levels of antioxidant enzymes (superoxide dismutase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase and ascorbate reductase) were much higher in habituated sugarbeet callus than in the normal form and has suggested (Hagege, 1996) that in general, habituated cells have very efficient oxidant scavenging systems. On the basis of changed metabolic patterns in habituated calluses including the high activity of antioxidant enzymes, accumulation of proline and polyamines reduction in tetrapyrroles and lipid peroxidation. Ledily *et al.,* (1993) proposed that such systems were in effect under permanent stress and they drew comparisons with animal cancer cells and vitrified tissue and later suggested (Gaspar *et al.,* 1998) that the changes serve to lock the cells in their habituated state.

18. THE TIMING AND DURATION OF GROWTH REGULATOR TREATMENTS

18.1. GROWTH REGULATOR EXPOSURE

It is common practice to vary the growth regulators to which tissues are exposed *in vitro*; for instance, callus is often initiated on a medium with one set of growth regulators and then moved to a second medium with different regulants for the induction of morphogenesis. Both the nature and the duration of the first and second treatments can be important and, because it is inconvenient to move explants frequently between one medium and another, recommended protocols are often a compromise between the most effective timings and durations of growth regulator exposure, and what is practical. Experimentation is complicated by the difficulty of determining growth regulator carry-over from one stage to another (for example, exogenously-applied auxins can become conjugated to other molecules from which they may be released when auxin is withdrawn from the medium), and the difficulty in determining the level of endogenous growth substances, (particularly cytokinins) which may be complementing, or antagonising exogenous treatments (Phillips, 1987).

The effect of the nature of the growth regulators in an induction medium, and the combined effects of concentration and timing, are well illustrated by some of the results of Behki and Lesley (1980). Tomato leaf disc explants were cultured for varying lengths of time with growth regulators, which induced callus formation. Small pieces of the callus (or the explant itself when transfer was effected after 1 day) were then moved to a medium containing other regulants known to be capable of inducing shoot formation. The results are illustrated in Fig. 7.6.

Whether there was continued callus growth, or the formation of adventitious shoots or roots, depended on the nature of the induction medium and the time at which transfer took place. Auxin is necessary early in the culture period to induce cell proliferation (Phillips, 1987).

18.2. PULSE TREATMENTS

When cultured material is exposed to a substance for only a short period of time, it is frequently said to have been given a pulse of that substance or regulant. Sometimes a pulse of a growth regulator at a relatively high rate can be as effective as a lower concentration, which is present continuously (Thomas and Tran-Van, 1982). Cotyledonary node explants of *Calocedrus decurrens*, for example, produced axillary shoots if exposed to 7-10 μM BA in the proliferation medium, or to 125 μM BA for 3 h (after which they were moved to a regulant-free medium) (Jelaska, 1987). Adventitious buds of *Picea alba* developed faster after a pulse of 250 μM BA for 2 h than if placed continually on a medium containing 5 μM BA (Von Arnold and Eriksson, 1985).

By supplying pulses of a growth regulator for progressively longer periods, it is possible to monitor the gaining of competence and the onset of determination in tissues. These processes can be further studied by the experimental application of metabolic inhibitors at specific times. Explants require to be exposed to a growth regulator for a minimum length of time for a given morphogenic event to be initiated.

Hussey (1977) showed that morphogenesis in *Freezia* could be determined by the duration of

exposure to growth regulators. When explants of soft stem tissue were placed for 12 h in a liquid culture medium containing 0.12 mg/l NAA and afterwards transferred to another medium without NAA but containing 0.5-1.0 mg/l BA, meristems were formed which gave rise directly to shoots. Increasing the duration of the preliminary NAA treatment had the same effect as increasing the concentration of NAA during an initial 12 h period, and induced the formation of callus having the capacity to form only roots. Continuous exposure of explanted tissue to NAA also caused the formation of callus, but its morphogenic capacity was determined by the concentration of the auxin: 0.015-0.03 mg/l NAA induced shoot-forming callus; continuous exposure to higher concentrations caused callus to be produced which mainly gave rise to roots.

Similarly thin cell layers from internodes of 2 week-old *Psophocarpus tetragonolobus* plants needed more than 5 days incubation with 1 µM IAA and 10 µM BA to become committed to produce adventitious shoots; but when exposed to the regulants for longer than 10 days, the tissues also produced callus and their capacity to form shoot buds declined (Hahn *et al.,* 1981; Table 7.2).

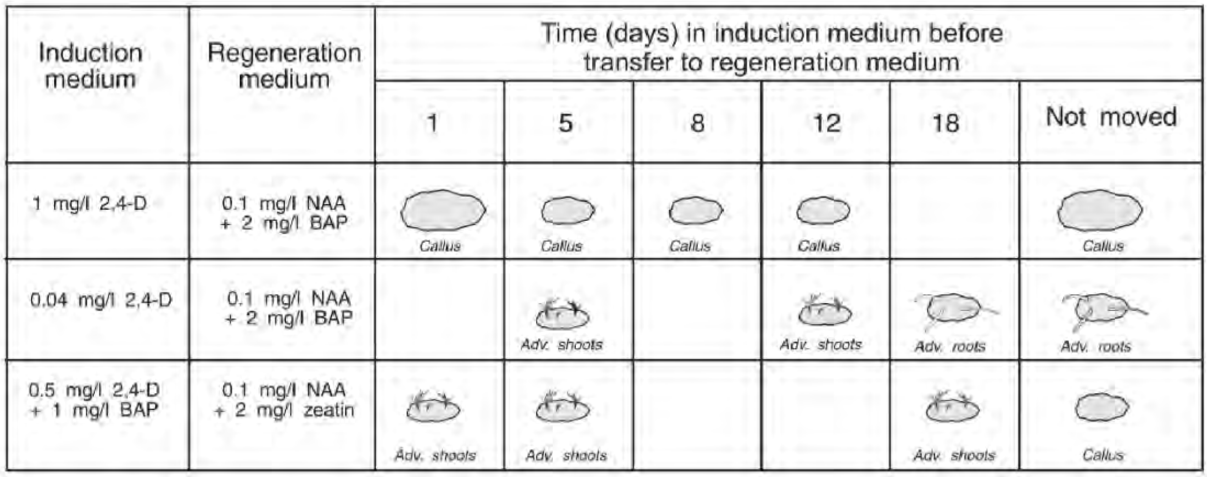


Fig. 7.6 The effect of the growth regulators used for callus induction, and the duration of this treatment, on subsequent morphogenesis. [From the data of Behki and Lesley (1980) – see text]

James and Wakerell (1982) found that apple shoots rooted optimally if cultured with 3 mg/l IBA for 4 days or 4.9 mg/l IAA for 6 days. Longer exposures decreased final root number and increased callus growth at the cut ends of the shoots. Margara (1969) found that the best treatment to induce direct adventitious shoots on *Brassica oleracea* var. *botrytis* inflorescence tissue, was to place the explants on a medium with 0.5 µM IBA for one week, and then transfer them to 10 µM for weeks 2 and 3.

Pinus strobus cotyledons required at least 4 days exposure to 4.4 µM BA for shoot formation to occur: 5 days exposure resulted in 50% of the explants responding, while after 7 days exposure there was an almost 100% response (Flinn and Webb, 1986).

Similarly *P. resinosa* embryos, or their cotyledons, needed to be exposed to 10-25 µM BA for at least 3 days for the stimulation of adventitious shoot formation (Noh *et al.,* 1988). Incubation for only 30-60 min on a medium containing 0.17 mg/l GA_3 was sufficient to partly inhibit shoot formation from dark grown tobacco callus. Repression was greatest when this treatment was applied during 6-10 days of incubation on a shoot-inducing medium. Abscisic acid could partly overcome the inhibitory effect, if instead of being placed in GA_3, the tissue was incubated in a GA_3 + ABA mixture for up to 30 min; after 30 min ABA failed to prevent the GA_3 repression (Thorpe and Meier, 1973).

18.4. EMBRYOGENESIS

The duration of auxin requirement has been most clearly determined for the induction of somatic embryogenesis, where it is now well established that an initial application must be withdrawn to permit embryo development. In carrot, 0.05 µM 2,4-D was required to be present for 6 days to induce competent single cells to form somatic embryos. Beyond this stage auxin was inhibitory (Komamine *et al.,* 1990).

Ghazi *et al.,* (1986) found that two types of embryogenic callus were produced from immature embryos and young seedling tissues of *Glycine max*

- a smooth and shiny callus which originated from the cotyledons; and
- a rough callus from immature embryos, cotyledon segments and germinated seedlings.

The rough callus took 8-9 weeks to form in the presence of 2-3 mg/l 2,4-D and 3 weeks to form with 5 mg/l 2,4-D. Smooth callus (which was the only kind from which plants were regenerated), was produced in 2 weeks using 10 mg/l 2,4-D.

Table 7.2 The result of exposing thin cell layers of *Psophocarpus* to IAA and BA for progressive lengths of time (data of Hahn *et al.,* 1981).

Days on medium with IAA and BA	Explants forming buds	Explants forming callus
0-5	0	0
7	100	0
10	100	0
15	100	100
25	40	100
45	20	100

19. OVER-EXPOSURE TO REGULANTS

The results above are concerned with cultured tissues gaining competence and becoming determined towards a developmental pathway. Over-exposure to a regulant through excessive concentration, or prolonged treatment, can result in a different developmental outcome to the one desired. The maximum number of adventitious shoots from *Datura innoxia* internodes, was obtained when they were pre-incubated with 1 μM 2,4-D for 6 days (haploid plants) or 12 days (diploid plants), before transfer to a shoot inducing medium (MS with 46.5 μM kinetin). Longer periods of pre-incubation resulted in less shoots being produced: there were none at all after 36 days (Forche *et al.,* 1981). Only two days of incubation with 0.1-10 mg/l 2,4-D was necessary to increase the number of buds regenerated from leaves of *Prunus canescens* (Antonelli and Druart, 1990).

Prolonging the exposure of cells to auxin, beyond the time necessary to induce embryogenesis, can result in the production of unregenerative callus, rather than embryogenic tissue.

REFERENCES

ABELES F.B., MORGAN O.W. & SALTVEIT M.E. 1992 *Ethylene in Plant Biology*. 2nd edition. San Diego, Academic Press 1992.
ADKINS S.W., SHIRAISHI T. & McCOMB J.A. 1990 Rice callus physiology – identification of volatile emissions and their effects on culture growth. Physiol. Plant. 78, 526-531.
ADUCCI P., BALLIO A., NASTA D., FOGLIANO V., FULLONE M.R. & MARRA M. 1996 Fusicoccin and its receptors: perception and signal transduction. Plant Growth Regul. 18, 93-98.
AKIMOTO C., AOYAGI H. & TANAKA H. 1999 Endogenous elicitor-like effects of alginate on physiological activities of plant cells. Appl. Microbiol. Biotechnol. 52, 429-436.
ALBERSHEIM P., DARVILL A.G., DAVIS K.R., DOARES S.H., GOLLIN D.J., O'NEILL R., TOUBART P.R. & YORK W.S. 1988 Oligosaccharins: regulatory molecules in plants. Hort-Science 23, 520.
ALCHANATIS V., PELEG K. & ZIV M. 1994 Morphological control and mensuration of potato plantlets from tissue cultures for automated micropropagation. Plant Cell Tissue Organ Cult. 36, 331-338.
ALDERSON P.G. & TAEB A.G. 1990 Influence of cultural environment of shoot growth and bulbing of tulip *in vitro*. Acta Hortic. 266, 91-94.
ALDWINCKLE H.S. & GUSTAFSON H.L. 1981 *In vitro* propagation of *Malus prunifolia* cultivar *xanthocarpa*. Env. Exp. Bot. 21, 440 .
AL-KHAYRI J. M. & AL-BAHRANY A.M. 2001 Silver nitrate and 2-isopentyladenine promote somatic embryogenesis in date palm (*Phoenix dactylifera* L.). Sci. Hortic. 89, 291-298.
ALONI R. 1980 Role of auxin and sucrose in the differentiation of sieve and tracheary elements in plant tissue cultures. Planta 150, 255-263.
ALTMAN A. & GOREN R. 1971 Promotion of callus formation by abscisic acid in citrus bud cultures. Plant Physiol. 47, 844-846.

ALTMAN A. & GOREN R. 1974 Growth and dormancy cycles in *Citrus* bud cultures and their hormonal control. Physiol. Plant. 30, 240-245.
ALTMAN A. & LEVIN N. 1993 Interactions of polyamines and nitrogen nutrition in plants. Physiol. Plant. 89, 653-658.
ALTMAN A. 1979 Inhibition of senescence of leaves and other plant organs by polyamines: possible mode of action. Isr. J. Bot. 28, 55-63.
ALTMAN A., NADEL B.L., FALASH F. & LEVIN N. 1990 Somatic embryogenesis in celery: induction, control and changes in polyamines and proteins. pp. 454-459 in Nijkamp *et al.,* (eds.) 1990 (q.v.).
AMMIRATO P.V. 1973 Some effects of abscisic acid on the development of embryos from caraway cells in suspension culture. Am. J. Bot. 60, 22.
AMMIRATO P.V. 1974 The effects of abscisic acid on the development of somatic embryos from cells of caraway (*Carum carvi* L.). Bot. Gaz. 135, 328-337.
AMMIRATO P.V. 1988 Role of ABA in regulation of somatic embryogenesis. HortScience 23, 520.
ANAND V.K., CHIBBAR R.N & NANDA K.K. 1972 Effects of GA_3 and IBA on rooting and on the sprouting of buds on stem cuttings of *Ipomoea fistulosa*. Plant Cell Physiol. 13, 912-917.
ANDERSON H.M., ABBOTT A.J. & WILTSHIRE S. 1982 Micropropagation of strawberry plants *in vitro* - effect of growth regulators on incidence of multi-apex abnormality. Sci. Hortic. 16, 331-341.
ANDERSON W.C. 1975 Propagation of rhododendrons by tissue culture. I. Development of a culture medium for multiplication of shoots. Comb. Proc. Int. Plant Prop. Soc. 25, 129-135.
ANDREAE W.A., VENIS M.A., JURSIC F. & DUMAS F. 1968 Does ethylene mediate root growth inhibition by indole-3-acetic acid? Plant Physiol. 43, 1375-1379.
ANTONELLI M. & DRUART PH. 1990 The use of brief 2,4-D treatment to induce leaf regeneration on *Prunus canescens* Bois. Acta Hortic. 280, 45-50.
APELBAUM A., BURGOON A.C., ANDERSON J.D., LIEBERMAN M., BEN-A-RIE R. & MATTOO A.K. 1981 Polyamines inhibit biosynthesis of ethylene in higher plant tissue and fruit protoplasts. Plant Physiol. 68, 453-456.
APELBAUM A., CANELLAKIS Z.N., APPLEWHITE B., KAUR-SAWHNEY R. & GALSTON A.W. 1988 Binding of spermidine to a unique protein in thin-layer tobacco tissue culture. Plant Physiol. 88, 996-998.
ASSMAN S.M. & ARMSTRONG F. 1999 Hormonal regulation of ion transporters: the guard cell system. pp. 337-362 in Hooykaas *et al.*, (eds.) 1999 (q.v.).
AZPEITIA A., CHAN J.L.. SAENZ L. & OROPEZA C. 2003. Effect of 22(S),23(S)-homobrassinolide on somatic embryogenesis in plumule explants of *Cocos nucifera* (L.) cultured *in vitro*. J. Hortic. Sci. Biotechnol. 78, 591-596.
BABIC V. & NESKOVIC M. 1984 Propagation of three blackberry cultivars from small apical buds *in vitro*. J. Hortic. Sci. 59, 183-185.
BACHMANN A.S., MATILE P. & SLUSARENKO A.J. 1998 Inhibition of ornithine decarboxylase activity by phaseolo-toxin: implications for symptom production in halo blight of French bean. Physiol. Mol. Plant Pathol. 53, 287-299.
BAGNI N. & BIONDI S. 1987 Polyamines. pp. 113-124 in Bonga J.M. and Durzan D.J. (eds.) 1987 *Cell and Tissue Culture in Forestry. Vol. 1. General Principles and Biotechnology*. Martinus Nijhoff Publishers, Dordrecht, Boston, Lancaster. ISBN 90-247-3430-4.
BAGNI N. 1966 Aliphatic amines and growth factor of coconut milk stimulating cellular proliferation of *Helianthus tuberosus* (Jerusalem artichoke) *in vitro*. Experimentia 22, 732.
BAGNI N. 1986 The function and metabolism of polyamines in plants. Acta Hortic. 179, 95-103.
BAGNI N., BARBIERI P. & TORRIGIANI P. 1983 Polyamine titer and biosynthetic enzymes during tuber formation in *Helianthus tuberosus*. J. Plant Growth Regul. 2, 177-184.
BAGNI N., CALZONI G.L. & SPERANZA A. 1978 Polyamines as sole nitrogen sources for *Helianthus tuberosus* explants *in vitro*. New Phytol. 80, 317-323.
BAJAJ S. & RAJAM M.V. 1995 Efficient plant regeneration from long-term callus cultures of rice by spermidine. Plant Cell Rep. 14, 717-720.
BAJAJ S. & RAJAM M.V. 1996 Polyamine accumulation and near loss of morphogenesis in long-term callus cultures of rice. Restoration of plant regeneration by manipulation of cellular polyamine levels. Plant Physiol. 112, 1343-1348.
BAPAT V.A. & RAO P.S. 1979 Somatic embryogenesis and plantlet formation in tissue cultures of sandalwood (*Santalum album*). Ann. Bot. 44, 629-632.
BARROSO J.B., CORPAS F.J., CARRERAS A., SANDALIO L.M., VALDERRAMA R., PALMA J.M., LUPIANEZ J.A. & DEL RIO L.A. 1999 Localization of nitric-oxide synthase in plant peroxisomes. J. Biol. Chem. 274, 36729-36733.
BARTELS P.G. & WATSON C.W. 1978 Inhibition of carotenoid synthesis by fluridone and norflurazon. Weed Sci. 26, 198-203.
BARZ W. & MACKENBROCK U. 1994 Constitutive and elicitation induced metabolism of isoflavones and pterocarpans in chikpea (*Cicer arietinum*) cell suspension cultures. Plant Cell Tissue Organ Cult. 38, 199-211.
BEASLEY C.A. & EAKS I.L. 1979 Ethylene from alclhol lamps and natural gas burners: effects on cotton ovule cultures *in vitro*. In Vitro 15, 263-269.
BEASLEY C.A. 1977 Ovule culture: fundamental and pragmatic research for the cotton industry. pp. 160-178 in Reinert and Bajaj (eds.) 1977 (q.v.).
BEHKI R.M. & LESLEY S.M. 1980 Shoot regeneration from leaf callus of *Lycopersicon esculentum*. Z. Pflanzenphysiol. 98, 83-87.
BELA J. & SHETTY K. 1999 Somatic embryogenesis in anise (*Pimpinella anisum* L.): the effect of proline on embryogenic callus formation and ABA on advanced embryo development. J. Food Biochem. 23, 17-32.
BELIGNI M.V. & LAMATTINA L. 2001a Nitric oxide: a non-traditional regulator of plant growth. Trends Plant Sci. 6, 508-509.
BELIGNI M.V. & LAMATTINA L. 2001b Nitric oxide in plants: the history is just beginning. Plant Cell Environ. 24, 267-278.
BENNICI A. & BRUSCHI P. 1999 Stability of habituation in *Nicotiana bigelovii* var. *bigelovii* shoots regenerated from habituated callus. Plant Sci. 143, 195-199.
BENNICI A. 1983 Expression of hormone-autotrophy in tissues of plantlets regenerated from habituated callus of *Nicotiana bigelovii*. Plant Cell Tissue Organ Cult. 2, 355-358.
BERETTA D. & ECCHER T. 1987 Determination of optimum level of BAP, GA_3 and IAA in micropropagation of *Camellia* by an orthogonal composite design. Acta Hortic. 212, 151-154.
BERGLUND T. & OHLSSON A.B. 1992 Effects of ethylene and aminoethoxyvinylglycine on cardenolide accumulation in tissue cultures of *Digitalis lanata*. J. Plant Physiol. 140, 395-398.
BERTOSSI F., BAGNI N., MORUZZI G. & CALDARERA C.M. 1965 Spermine as a new growth-promoting substance for *Helianthus tuberosus* (Jerusalem artichoke) *in vitro*. Experimentia 21, 80-81.
BEYER E.M. 1976a Silver ion: a potent antiethylene agent in cucumber and tomato. HortScience 11, 195-196.

BEYER E.M. 1976b A potent inhibitor of ethylene action in plants. Plant Physiol. 58, 268-271.

BEYER E.M. 1979 Effects of silver ion, carbon dioxide and oxygen on ethylene action and metabolism. Plant Physiol. 63, 169-173.

BEYER E.M. 1985 Ethylene metabolism. pp. 125-137 in Roberts J.A. and Tucker J.A. (eds.) *Ethylene and Plant Development*. Butterworths, London

BIDDINGTON N.L., SUTHERLAND R.A. & ROBINSON H.T. 1988 Silver nitrate increases embryo production in anther culture of Brussels sprouts. Ann. Bot. 62, 181-185.

BIGOT C. & NITSCH J.P. 1968 Effet du moment de l'aplication d'acide gibbérellique ou de 2,6-diaminopurine sur la néoformation des bourgeons chez *Begonia rex* Putz. Compt. Rend. Acad. Sci. Paris 267D, 619-621.

BINNS A. & MEINS F. Jr. 1979 Cold-sensitive expression of cytokinin habituation by tobacco pith cells in culture. Planta 145, 365-369.

BIONDI S., BAGNI N. & SANSOVINI A. 1986 Dicyclohexylamine uptake and effects on polyamine content in cultured cotyledons of radiata pine. Physiol. Plant. 66, 41-45.

BIONDI S., SCARAMAGLI S., CAPITANI F., ALTAMURA M.M. & TORRIGIANI P. 2001 Methyl jasmonate upregulates biosynthetic gene expression, oxidation and conjugation of polyamines, and inhibits shoot formation in tobacco thin layers. J. Exp. Bot. 52, 231-242.

BISBIS B., KEVERS C., DOMMES J. & GASPAR TH. 2000a Interactions between polyamine and ethylene metabolisms in a hormone autonomous sugarbeet callus. J. Plant Physiol. 157, 24-30.

BISBIS B., WAGNER A.M., KEVERS C. & GASPAR TH. 2000b A comparison of respiratory pathways in fully habituated and normal non-organogenic sugarbeet callus. J. Plant Physiol. 156, 312-318.

BLACKMON W.J. & REYNOLDS B.D. 1982 *In vitro* shoot regeneration of *Hibiscus acetosella*, muskmelon, watermelon and winged bean. HortScience 17, 588-589.

BLACKMON W.J., REYNOLDS B.D. & POSTEK C.E. 1981a Regeneration of shoots from cantaloupe, crepe myrtle, rosells and winged bean explants. Environ. Exp. Bot. 21, 438-439.

BLACKMON W.J., REYNOLDS B.D. & POSTEK C.E. 1981b Production of somatic embryos from callused cantaloupe hypocotyls explants. HortScience 16, 451.

BLEECKER A.B. 1999 Ethylene perception and signalling: an evolutionary perspective. Trends Plant Sci. 4, 269-274.

BLUMENFELD A. & GAZIT S. 1970 Interaction of kinetin and abscisic acid in the growth of soybean callus. Plant Physiol. 45, 535-536.

BONGA J.M. & DURZAN D.J (eds.) 1982 *Tissue Culture in Forestry*. Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, Boston, Lancaster. ISBN 90-247-2660-3.

BOSTRACK J.M. & STRUCKMEYER B.E. 1967 Effect of gibberellic acid on growth and anatomy of *Coleus blumei*, *Antirrhinum majus* and *Salvia splendens*. New Phytol. 66, 539-544.

BOUCHEREAU A., AZIZ A., LARHER F. & MARTIN-TANGUY J. 1999 Polyamines and environmental challenges: recent development. Plant Sci. 140, 103-125.

BOULAY M. & FRANCLET A. 1986 Dégénérescence clonal du Douglas (*Pseudotsuga menziesii*) cultivé *in vitro*; relation avec la teneur en éléments mineraux, en auxine, acide abscissique, zéatine et zéatine-riboside. Compt. Rend. Acad. Ci. Paris 303, ser. 111.

BOURGIN J.-P. & NITSCH J.P. 1969 Production of haploid *Nicotiana* from excised stamens. Ann. Physiol. Veg. 9, 377-382.

BOXUS PH. 1974a The production of strawberry plants by *in vitro* micro-propagation. J. Hortic. Sci. 49, 209-210.

BOXUS PH. 1974b La micropropagation '*in vitro*' du fraisier. Proc. Int. Hort. Cong. 19, (1a)65.

BOXUS PH., QUOIRIN M. & LAINE J.M. 1977 Large-scale propagation of strawberry plants from tissue culture. pp. 130-143 in Reinert and Bajaj (eds.) 1977 (q.v.).

BRADLEY D.J., KJELLBOM P. & LAMB C.J. 1992 Elicitor-induced and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein - a novel, rapid defence response. Cell 70, 21-30.

BRADLEY P.M., EL-FIKI F. & GILES K.L. 1984 Polyamines and arginine affect somatic embryogenesis of *Daucus carota*. Plant Sci. Lett. 34, 397-401.

BRADLEY P.M., EL-FIKI F. & GILES K.L. 1985 The effects of putrescine on somatic embryogenesis of *Daucus carota* as examined by two-dimensional electrophoresis. pp. 307-308 in Henke *et al.,* (eds.) 1985 (q.v.).

BRESSAN P.H., KIM Y.-J., HYDMAN S.E., HASEGAWA P.M. & BRESSAN R.A. 1982 Factors affecting *in vitro* propagation of rose. J. Am. Soc. Hortic. Sci. 107, 979-990.

BRIAN P.W. 1959 Effects of gibberellins on plant growth and development. Biol. Rev. 34, 37-84.

BRIDGEN M.P. & LINEBERGER R.D. 1981 The effect of hormones on the ethylene production rate of *Nicotiana tabacum* 'KY 10' callus *in vitro*. HortScience 16, 441.

BROSA C. 1999 Biological effects of brassinosteroids. Crit. Rev. Biochem. Mol. Biol. 34, 339-358.

BROWN D.J., CANVIN D.T. & ZILKEY B.F. 1970 Growth and metabolism of *Ricinus communis* endosperm in tiussue culture. Can. J. Bot. 48, 2323-2331.

BROWN R.G.S., KAWAIDE H., YANG Y.Y., RADEMACHER W. & KAMIYA Y. 1997 Daminozide and prohexadione have similar modes of action as inhibitors of the late stages of gibberellin metabolism. Physiol. Plant. 101, 309-313.

BUCHALA A.J. & SCHMID A. 1979 Vitamin D and its analogues as a new class of plant growth substances affecting rhizogenesis. Nature 280, 230-231.

BUFLER G. 1986 Ethylene-promoted conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene in peel of apple at various stages of fruit development. Plant Physiol. 80, 539-543.

BURG S.P. & BURG E.A. 1967 Molecular requirements for the biological activity of ethylene. Plant Physiol. 42, 144-152.

BUSK P.K., PUJAL J., JESSOP A., LUMBRERAS V. & PAGES M. 1999 Constitutive protein-DNA interactions on the abscisic acid-responsive element before and after developmental activation of the *rab28* gene. Plant Mol. Biol. 41, 529-536.

BUTTON J. & KOCHBA J. 1977 Tissue culture in the citrus industry. pp. 70-92 in Reinert and Bajaj (eds.) 1977 (q.v.)

BUTTON J. KOCHBA J. & BORNMAN C.H. 1974 Fine structure of and embryoid development from embryogenic ovular callus of 'Shamouti' orange (*Citrus sinensis* Osb.). I. Exp. Bot. 25, 446-457.

BYUN S.Y., RYU Y.W., KIM C. & PEDERSEN H. 1992 Elicitation of sanguinarine production in two-phase cultures of *Eschscholtzia californica*. J. Ferment. Bioeng. 73, 380-385.

CAMLOH M., RAVNIKAR M. & ZEL J. 1996 Jasmonic acid promotes division of fern protoplasts, elongation of rhizoids and early development of gametophytes. Physiol. Plant. 97, 659-664.

CAMLOH M., VILHAR B., ZEL J. & RAVNIKAR M. 1999 Jasmonic acid stimulates development of rhizoids and shoots in fern leaf culture. J. Plant Physiol. 155, 798-801.

CANHOTO J.M., LOPES M.L. & CRUZ G.S. 1999 Somatic embrogenesis and plant regeneration in myrtle (Myrtceae). J. Cell

Tiss. Org. Cult. 53(1), 13-21.
CAPUANA M. & DEBERGH P.C. 1997 Improvement of the maturation and germination of horse chestnut somatic embryos. Plant Cell Tisue Organ Cult. 48, 23-29.
CASTILLO B., SMITH M.A.L. & YADAVA U.L. 1998 Liquid system scale up of *Carica papaya* L. somatic embryogenesis. J. Hort. Sci. Biotech. 73, 307-311.
CHADWICK A.V. & BURG S.P. 1967 An explanation of the inhibition of root growth caused by indole-3-acetic acid. Plant. Physiol. 42, 415-420.
CHALUTZ E. & DEVAY J.E. 1969 The production of ethylene *in vitro* and *in vivo* by *Ceratocystis fimbriata* in relation to disease development. Phytopath. 59, 750-755.
CHANG W.C. & HSING Y.-I. 1980a *In vitro* flowering of embryoids derived from mature root callus of ginseng (*Panax ginseng*). Nature 284, 341-342.
CHANG W.C. & HSING Y.-I. 1980b Plant regeneration through somatic embryogenesis in root-derived callus of ginseng (*Panax ginseng* C.A. Meyer). Theor. Appl. Genet. 57, 133.
CHAO Q., ROTHENBERG M., SOLANO R., ROMAN G., TERZAGHI W. & ECKER J. 1997 Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell 89, 1133-1144.
CHARRIERE F., SOTTA B., MIGINIAC E. & HAHNE G. 1999 Induction of adventitious shoots or somatic embryos on *in vitro* cultured zygotic embryos of *Helianthus annuus*: variation of endogenous hormone levels. Plant Physiol. Biochem 37, 751-757.
CHATTOPADHYAY M.K. & GHOSH B. 1998 Molecular analysis of polyamine biosynthesis in higher plants. Curr. Sci. 74, 517-522.
CHEN, J. T. & CHANG W. C. 2003. 1-aminocyclopropane-1-carboxylic acid enhanced direct somatic embryogenesis from *Oncidium* leaf cultures. Biol. Plant. 46, 455-458.
CHENG T.-Y. 1972 Induction of IAA synthesis in tobacco pith explants. Plant Physiol. 50, 723-727.
CHEONG, E. J. & POOLER, M. R. 2004. Factors affecting somatic embryogenesis in *Prunus incisa* cv. February Pink. Plant Cell Rep. 11, 810-815
CHI G.-L., BARFIELD D.G., SIM G.-E. & PUA E.-C. 1990 Effect of $AgNO_3$ and aminoethoxyvinilglycine on *in vitro* shoot and root organogenesis from seedling explants of recalcitrant Brassica genotypes. Plant Cell Rep. 9, 195-198.
CHLYAH A. 1972 Néoformation caulinaire et radiculaire chez les fragments de feuilles de *Begonia rex* Putz: Action de divers facteurs régulateurs trophiques et d'environment. Biol Plant 14, 204-212.
CHO S.-C. 1983 Effects of cytokinin and several inorganic cations on the polyamine content in lettuce cotyledons. Plant Cell Physiol. 24, 27-32.
CHRAIBI K.M., LATCHÉ A., ROUSTAN J.P. & FALLOT J. 1991 Stimulation of shoot regeneration from cotyledons of *Helianthus annuus* by the ethylene inhibitors, silver and cobalt. Plant Cell Rep. 10, 204-207.
CLOUSE S.D. & SASSE J.M. 1998 Brassinosteroids: essential regulators of plant growth and development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 427-451.
COLCLASURE G.C. & YOPP J.H. 1976 Galactose-induced ethylene evolution in mung bean hypocotyls: a possible mechanism for galactose retardation of plant growth. Physiol. Plant. 37, 298-302.
COLEMAN W.K. & GREYSON R.I. 1977a Analysis of root formation in leaf discs of *Lycopersicon esculentum* Mill. cultured *in vitro*. Ann. Bot. 41, 307-320.
COLEMAN W.K. & GREYSON R.I. 1977b Promotion of root initiation by gibberellic acid in leaf discs (*Lycopersicon esculentum*) cultured *in vitro*. New Phytol. 78, 47-54.
COLEMAN W.K. & THORPE T.A. 1977 *In vitro* culture of Western Red Cedar (*Thuja plicata* Donn) 1. Plantlet formation. Bot. Gaz. 138, 298-304.
COLEMAN W.K., HUXTER T.J., REID D.M. & THORPE T.A. 1980 Ethylene as an endogenous inhibitor of root regeneration in tomato leaf discs cultured *in vitro*. Physiol. Plant. 48, 519-525.
CONSTANTIN M.J., HENKE R.R. & MANSUR M.A. 1976 Effects of activated charcoal and and hormones on growth and organogenesis in tobacco callus. In Vitro, 12, 332-333.
CONSTANTIN M.J., HENKE R.R. & MANSUR M.A. 1977 Effects of activated charcoal on callus growth and shoot organogenesis in tobacco. In Vitro 13, 293-296.
COUMANS M., COUMANS-GILLÈS M.F., MENARD D., KEVERS C. & CEULEMANS E. 1982 Micropropagation of sugarbeet: possible ways. pp. 689 in Fujiwara (ed) 1982 (q.v.).
COUMANS-GILLÈS M.F., COUMANS M., CEULEMANSE E. & GASPAR TH. 1981 Vegetative multiplication of sugarbeet through *in vitro* culture of inflorescence pieces. Plant Cell Tissue Organ Cult. 1, 93-101.
CREISSEN G.P. & KARP A. 1985 Karyotypic changes in potato plants regenerated from protoplasts. Plant Cell Tissue Organ Cult. 4, 171-182.
CVIKROVA M., MALA J., EDER J., HRUBCOVA M. & VAGNER M. 1998 Abscisic acid, polyamines and phenolic acids in sessile oak somatic embryos in relation to their conversion potential. Plant Physiol. Biochem. 36, 247-255.
DAGNINO D.S., CARELLI M.L.D., ARRABAL R.F. & ESQUIBEL M.A. 1991 Effect of gibberellic acid on *Ipomoea batatas* regeneration from meristem culture. Pesq. Agrop. Bras. 26, 259-262.
DAI J. & CAMPBELL W.F. 1981 Response of tomato plants to stressful temperatures increase in abscisic acid concentrations. Plant Physiol. 67, 26-29.
DAI J., KAUR-SAWHNEY R. & GALSTON A.W. 1982 Promotion by gibberellic acid of polyamine biosynthesis in internodes of light-grown dwarf peas. Plant Physiol. 69, 103-105.
DALESSANDRO G. 1973 Interaction of auxin, cytokinin and gibberellin on cell division and xylem differentiation in cultured explants of Jerusalem artichoke. Plant Cell Physiol. 14, 1167-1176.
DALTON C.C. & STREET H.E. 1976 The role of the gas phase in the greening and growth of illuminated cell suspension cultures of spinach (*Spinacea oleracea* L.). In Vitro 12, 485-494.
DAS A.B., ROUT G.R. & DAS P.P. 1995 *In vitro* somatic embryogenesis from callus-culture of the timber-yielding tree *Hardwickia binata* Roxb. Plant Cell Rep. 15, 147-149.
DAVIDONIS G.H. 1990 Gibberellic acid-induced cell elongation in cotton suspension cultures. J. Plant Growth Regul. 9, 243-246.
DAVIS D.G. 1997 2,4-Dichlorophenoxy acetic acid and indole-acetic acid partially counteract inhibition of organo-genesis by difluoromethylornithine. Physiol. Plant 101, 425-433.
DAVIS T.D., STEFFENS G.L. & SANKHLAN N. 1988 Triazole plant growth regulators. Hortic. Rev. 10, 63-105.
DE FOSSARD R.A. & DE FOSSARD H. 1988 Micropropagation of some members of the Myrtaceae. Acta Hortic. 227, 346-351.
DE GREEF W. & JACOBS M. 1979 *In vitro* culture of the sugarbeet: description of a cell line with high regeneration capacity. Plant Sci. Lett. 17, 55-61.
DE LANGHE E. & DE BRUIJNE E. 1976 Continuous propagation of tomato plants by means of callus cultures. Sci. Hortic. 4, 221-227.

DE PROFT M.P., MAENE M.L. & DEBERGH P. 1985 Carbon dioxide and ethylene evolution in the culture atmosphere of *Magnolia* cultured *in vitro*. Physiol. Plant. 65, 375-379.

DE JONG A.J., HEIDSTRA R., SPAINK H.P., HARTOG M.V., MEIJER E.A., HENDRIKS T., LOSCHIAVO F., TERZI M., BISSELING T., VANKAMMEN A. & DE VRIES S.C. 1993 *Rhizobium* lipooligosaccharides rescue a carrot somatic embryo mutant. Plant Cell 5, 615-620.

DELAMOMARCO J.B. & PICAZO I. 1994 Effect of growth regulators on *in vitro* propagation of *Ficus benjamina* cv. Exotica. Biol. Plant. 36, 167-173.

DELBREIL B. & JULLIEN M. 1994 Evidence for *in vitro* induced mutation which improves somatic embryogenesis in *Asparagus officinalis* L. Plant Cell Rep. 13, 372-376.

DELLEDONNE M., XIA Y.J., DIXON R.A. & LAMB C. 1998 Nitric oxide functions as a signal in plant disease resistance. Nature 394, 585-588.

DODEMAN V.L., DUCREUX G. & KREIS M. 1997 Zygotic embryogenesis versus somatic embryogenesis. J. Exp. Bot. 48, 1493-1509.

DORLEY D. & LEYTON L. 1968 Effects of growth regulating substances and water potential on the development of secondary xylem in *Fraxinus*. New Phytol. 67, 579-594.

DRUART PH. & DEWULF O. 1993 Activated charcoal catalyzes sucrose hydrolysis during autoclaving. Plant Cell Tissue Organ Cult. 32, 97-99.

DRUART PH. 1988 Regulation of axillary branching in micro-propagation of woody fruit species. Acta Hortic. 227, 369-380.

DUNCAN D.R. & WIDHOLM J.M. 1987 Improved plant regeneration from maize callus cultures using $AgNO_3$. Plant Physiol. 83, Suppl. 35,208.

DUNCAN D.R. & WIDHOLM J.M. 1988 Improved plant regeneration from maize callus cultures using 6-benzyl-aminopurine. Plant Cell Rep. 7, 452-455.

DUNSTAN D.I., BEKKAOUI F., FILON M., FOWKE L.C. & ABBRAMS S.R. 1988 Effects of abscisic acid and analogues on the maturation of white spruce (*Picea glauca*) somatic embryos. Plant Sci. 58, 77-84.

DUNWELL J.M. 1979 Anther culture in *Nicotianum tabacum*: the role of the culture vessel atmosphere in pollen embryo induction and growth. J. Exp. Bot. 30, 419-428.

DURNER J., SHAH J. & KLESSIG D.F. 1997 Salicylic acid and disease resistance in plants. Trends Plant Sci. 2, 266-274.

EILERS R.J., SULLIVAN J.C. & SKIRVIN R.M. 1988 Analyzing the effects of exogenous polyamines and growth regulators on plating efficiency of sweet potato protoplasts using a central composite test design. Plant Cell Rep. 7, 216-219.

ELLIOTT R.F. 1970 Axenic culture of meristem tips of *Rosa multiflora*. Planta 95, 183-186.

ELMER O.H. 1936 Growth inhibition in the potato caused by a gas emanating from apples. J. Agric. Res. 52, 609-626.

ELMORE H.W. & WHITTIER D.P. 1973 The role of ethylene in the induction of apogamous buds in *Pteridium* gametophytes. Planta 111, 85-90.

EMONS A.M.C., SAMALLO-DROPPERS A. & VAN DER TOORN C. 1993 The influence of sucrose, mannitol, L-proline, abscisic acid and gibberellic acid on the maturation of somatic embryos of *Zea mays* L. from suspension cultures. J. Plant Physiol. 142, 597-604.

ENGELKE A.L., HAMZI H.Q. & SKOOG F. 1973 Cytokinin-gibberellin regulation of shoot development and leaf form in tobacco plantlets. Am. J. Bot. 60, 491-495.

ERNST R. 1974 The use of activated charcoal in asymbiotic seedling culture of *Paphiopedilum*. Am. Orchid Soc. Bull. 43, 35-38.

EVANARI M. 1961 Chemical influences of other plants (allelopathy). pp. 691-736 in Ruhland W.(ed.) 1961 *Aussen-factoren in Wachstum und Entwicklung*. Springer, Berlin

EVANS D.A., SHARP W.R. & PADDOCK E.F. 1976 Variation in callus proliferation and root morphogenesis in leaf tissue cultures of *Glycine max* strain T 219. Phytomorph. 26, 379-384.

EVANS P.T. & MALMBERG R.L. 1989 Do polyamines have roles in plant development? Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 235-269.

EVEN-CHEN Z., MATTOO A.K. & GOREN R. 1982 Inhibition of ethylene biosynthesis by aminoethoxyvinylglycine and by polyamines shunts label from 3,4-^{14}C-methionine into spermidine in aged orange peel discs. Plant Physiol. 69, 385-388.

FABIJAN D., TAYLOR J.S. & REID D.M. 1981 Adventitious rooting in hypocotyls of sunflower (*Helianthus annuus*) seedlings. II. Action of gibberellins, cytokinins, auxins and ethylene. Physiol. Plant. 53, 589-597.

FAURE O., DEWITTE W., NOUGAREDE A. & VAN ONCKELEN H. 1998 Precociously germinating somatic embryos of *Vitis vinifera* have lower ABA and IAA levels than their germinating zygotic counterparts. Physiol. Plant. 102, 591-595.

FEIRER R., MIGNON G. & LITVAY J. 1984 Arginine decarboxylase and polyamines required for embryogenesis in the wild carrot. Science 223, 1433-1435.

FEIRER R.P., WANN S.R. & EINSPHAR D.W. 1985 The effects of spermidine synthesis inhibitors on *in vitro* plant development. Plant Growth Regul. 3, 319-327.

FELIX G. & BOLLER T. 1995 Systemin induces rapid ion fluxes and ethylene biosynthesis in *Lycopersicon peruvianum* cells. Plant J. 7, 381-389.

FERNANDEZ S., MICHAUX-FERRIERE N. & COUMANS M. 1999 The embryogenic response of immature embryo cultures of durum wheat (*Triticum durum* Desf.): histology and improvement by $AgNO_3$. Plant Growth Regul. 28, 147-155.

FERNANDO S.C. & GAMAGE C.K.A. 2000 Abscisic acid induced somatic embryogenesis in immature embryo explants of coconut (*Cocos nucifera* L.). Plant Sci. 151, 193-198.

FIDLER J.C. 1960 Naturally occurring volatile organic compounds. pp. 347-359 in Encyclopedia of Plant Physiology 12(2) Springer-Verlag, Berlin.

FIENBERG A.A., CHOI J.H., LUBICH W.P. & SUNG Z.R. 1984 Developmental regulation of polyamine metabolism in growth and differentiation of carrot culture. Planta 162, 532-539.

FIORE M.C., TRABACE T. & SUNSERI F. 1997 High frequency of plant regeneration in sunflower from cotyledons via somatic embryogenesis. Plant Cell Rep. 16, 295-298.

FLETCHER R.A., ASARE-BOAMAH N.K., KRIEG L.C., HOFSTRA G. & DUMBROFF E.B. 1988 Triadimefon stimulates rooting in bean hypocotyls. Physiol. Plant. 73, 401-405.

FLINN B. & WEBB D.T. 1986 Effects of media components and the timing of cytokinin application on caulogenesis from *Pinus strobes* embryonic explants. p. 36 in Abst. VI Int. Congr. Plant Tissue & Cell Culture, Minneapolis, USA.

FLORES H.E. & GALSTON A.W. 1984 Osmotic stress-induced polyamine accumulation in cereal leaves: I. Physiological parameters of the response. Plant Physiol. 75, 102-113.

FOBERT P.R. & WEBB D.T. 1988 Effects of polyamines, polyamine precursors and polyamine biosynthetic inhibitors on somatic embryogenesis from eggplant (*Solanum melongena*) cotyledons. Can. J. Bot. 66, 1556-1565.

FORCHE E., KIBLER R. & NEUMANN K.-H. 1981 the influence of developmental stages of haploid and diploid callus cultures of *Datura innoxia* on shoot initiation. Z. Pflanzenphysiol. 101, 257-262.

FORD Y.Y., RATCLIFFE R.G. & ROBINS R.J. 1998 *In vivo* nuclear magnetic resonance analysis of polyamine and alkaloid

metabolism in transformed root cultures of *Datura stramonium* L.: evidence for the involvement of putrescine in phytohormone-induced de-differentiation. Planta 205, 205-213.
FRETT J.J. & SMAGULA J.M. 1981 Effect of stock plant pretreatment on lowbush blueberry explant shoot multiplication. HortScience 16, 418.
FRIDBORG G. & ERIKSSON T. 1975 Effects of activated charcoal on growth and morphogenesis in cell cultures. Physiol. Plant. 34, 306-308.
FRIDBORG G., PEDERSEN M., LANDSTROM M. & ERIKSSON T. 1978 The effect of activated charcoal on tissue cultures: absorbtion of metabolites inhibiting morphogenesis. Physiol. Plant. 43, 104-106.
FRIEDMAN R., ALTMAN A. & BACHRACH U. 1982 Polyamines and root formation in mung bean hypocotyls cuttings. I. Effect of endogenous compounds on endogenous polyamic content. Plant Physiol. 70, 844-848.
FRIEDMAN R., ALTMAN A. & BACHRACH U. 1985 Polyamines and root formation in mung bean hypocotyls cuttings. II. Incorporation of precursors into polyamines. Plant Physiol. 79, 80-83.
FUJIMURA T. & KOMAMINE A. 1975 Effects of various growth regulators on the embryogenesis in a carrot cell suspension culture. Plant Sci. Lett. 5, 359-364.
FUJIOKA S., LI J.M., CHOI Y.H., SETO H., TAKATSUTO S., NOGUCHI T., WATANABE T., KURIYAMA H., YOKOTA T., CHORY J. & SAKURAI A. 1997 The *Arabidopsis* deetiolated2 mutant is blocked early in brassinosteroid biosynthesis. Plant Cell 9, 1951-1962.
FUJIOKA S.T., NOGUCHI T., WATANAB T., TAKATSUTO S. & YOSHIDA S. 2000 Biosynthesis of brassinosteroids in cultured cells of *Catheranthus roseus*. Phytochemistry 53, 549-553.
FUJIWARA A. (ed.) 1982 *Plant Tissue Culture* 1982. Proc. 5th. Int. Cong. Plant Tiss. Cell Cult., Japan. Assoc. Plant Tissue Culture, Japan.
GALSTON A.W. 1983 Polyamines as modulators of plant development. Biosci. 33, 382-388.
GALSTON A.W., ALTMAN A. & KAUR-SAWHNEY R. 1978 Polyamines, ribonuclease and the improvement of oat leaf protoplasts. Plant Sci. Lett. 11, 69-79.
GALSTON A.W., DAI Y.-R., FLORESH H. & YOUNG N.D. 1983 The control of arginine decarboxylase activity in higher plants. pp. 381-393 in Bachrach *et al.,* (eds.) 1983 *Advances in Polyamine Research* 4.
GALSTON A.W., SAWHNEY R.K., ALTMAN A. & FLORES H. 1980 Polyamines, macromolecular synthesis and the problem of cereal protoplast regeneration. pp. 485-497 in Ferenczy I. and Farkas G.L. (eds.) 1980 *Advances in Protoplast Research.* Proc. 5th Int. Protoplast Symposium. Szeged, Hungary 1979. Permagon Press, Oxford, New York, Toronto.
GAMBORG O.L. & LARUE T.A.G. 1968 Ethylene produced by plant cells in suspension cultures. Nature 220, 604-605.
GAMBORG O.L. & LARUE T.A.G. 1971 The effect of auxins, abscisic acid and kinetin on ethylene production in suspension cultures of rose and *Ruta* cells. Plant Physiol. 48, 399-401.
GAMBORG O.L., DAVIS B.P. & STAHLUT R.W. 1983 Somatic embryogenesis in cell cultures of *Glycine* species. Plant Cell Rep. 2, 209-212.
GARCIA F. & EINSET J.W. 1982 Ethylene and ethane production in salt-stressed or 2,4-D-stressed tobacco tissue cultures. In Vitro 18, 275.
GARCIA F. & EINSET J.W. 1983 Ethylene and ethane production in 2,4-D-treated and salt-treated tobacco tissue cultures. Ann. Bot. 51, 287-295.
GASPAR TH., BISBIS B., KEVERS C., PENEL C., GREPPIN H., LEDILY F., BILLARD J.P., HUAULT C., GARNIER F., RIDEAU M. & FOIDART J.M. 1998 Atypical metabolisms and biochemical cycles imposing the cancerous state on plant cells. Plant Growth Regul 24, 135-144.
GASPAR Th., HAGEGE D., KEVERS C., PENEL C., CREVECOEUR M., ENGELMANN I., GREPPIN H. & FOIDART J.M. 1991 When plant teratomas turn into cancers in the absence of pathogens. Physiol. Plant 83, 696-701.
GAUTAM V.K., MITTAL A., NANDA K. & GUPTA S.C. 1983 *In vitro* regeneration of plantlets from somatic explants of *Matthiola incana*. Plant Sci. Lett. 29, 25-32.
GAUTHERET R.J. 1961 Action conjuguée de l'acide gibbérellique, de la cinétine et de l'acide-acétique sur les tissus cultivés *in vitro*, particulièrement sur ceux de Tropinambour. Compt. Rend. Acad. Sci. Paris 253, 1381-1385.
GAUTHERET R.J. 1969 Investigations on the root formation in the tissue of *Helianthus tuberosus* cultured *in vitro*. Am. J. Bot. 56, 702-717.
GAVIDIA I., ZARAGOZA C., SEGURA J. & PEREZ-BERMUDEZ P. 1997 Plant regeneration from juvenile and adult *Anthyllis cytisoides*, a multipurpose leguminous shrub. J. Plant Physiol. 150, 714-718.
GAVINLERTVATANA P., READ P.E., WILKINS H.F. & HEINS R. 1982 Ethylene levels in flask atmospheres of *Dahlia pinata* Cav. leaf segments and callus cultured *in vitro*. J. Am. Soc. Hortic. Sci. 107, 3-6.
GAWRONSKA H., BURZA W., BOLESTA E. & MALEPSZY S. 2000 Zygotic and somatic embryos of cucumber (*Cucumis sativus* L.) substantially differ in their levels of abscisic acid. Plant Sci. 157, 129-137.
GHAZI T.D., CHEEMA H.V. & NABORS M.W. 1986 Somatic embryogenesis and plant regeneration from embryogenic callus of soybean, *Glycine max* L. Plant Cell Rep. 5, 452-456.
GHOSH B. & SEN S. 1991 Plant regeneration through somatic embryogenesis from spear callus culture of *Asparagus cooperi* Baker. Plant Cell Rep. 9, 667-670.
GIBSON, S. I. 2004. Sugar and phytohormone response pathways: navigating a signalling network. J. Exp. Bot. 55, 253-264.
GONZALEZ A., ARIGITA L., MAJADA J. & TAMES R.S. 1997 Ethylene involvement in *in vitro* organogenesis and plant growth of *Populus tremula* L. Plant Growth Regul. 22, 1-6.
GOODWIN P.B., KIM Y.C. & ADISARWANTO T. 1980 Propagation of potato by root-tip culture. 1. Shoot multiplication. Potato Res. 23, 9-18.
GRANTZ A., BRUMMELL D.A. & BENNETT A.B. 1995 Ascorbate free radical reductase messenger RNA levels are induced by wounding. Plant Physiol. 108, 411-418..
GUERN J. 1987 Regulation from within: the hormone dilemma. Ann. Bot. 60, 75-102.
GUEVIN T.G. & KIRBY E.G. 1997 Induction of embryogenesis in cultured mature zygotic embryos of *Abies fraseri* (Pursh.) Poir. Plant Cell Tissue Organ Cult. 49, 219-222.
GUNDLACH H., MULLER M.J., KUTCHAN T.M. & ZENK M.H. 1992 Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc. Natl. Acad. Sci. USA 89, 2389-2393.
GUO Z.J. & OHTA Y. 1994 Effect of ethylene biosynthesis on the accumulation of 6-methoxymellein induced by elicitors in carrot cells. J. Plant Physiol. 144, 700-704.
HAGEGE D. 1996 Habituation in sugarbeet plant cells: permanent stress or antioxidant adaptative strategy? In Vitro Cell. Dev. Biol.-Plant 32, 1-5.
HAGEGE D., KEVERS C., SALABERT P. & GASPAR TH. 1992 Protective systems against activated oxygen species compared in

normal and fully habituated non-organogenic sugarbeet calluses. In Vitro Cell. Dev. Biol.-Plant 28P, 143-147.
HAHN M.G. 1996 Microbial elicitors and their receptors in plants. Annu. Rev. Phytopathol. 34, 387-412.
HAHN T.T., SCHRICKE H.L. & VAN K.T.T. 1981 Direct *in vitro* bud formation from fragments and thin cell layers of different organs of the winged bean (*Psophocarpus tetragonolobus* L. DC.). Z. Pflanzenphysiol. 102, 127-139.
HALL M.A., ACASTER M.A., BENGOCHEA T., DODDS J.H., EVANS D.E., JONES J.F., JERIE P.H., MUTUMBA G.C., NIEPEL B. & SHAARI A.R. 1980 Ethylene and seeds. pp. 199-207 in Skoog (ed.) 1980 (q.v.).
HALL M.A., NOVIKOVA G.V., MOSHKOV I.E., MUR L.A.J. & SMITH A.R. 2002 Protein kinases in plants in the transduction of abiotic and biotic signals. Russ. J. Plant Physiol. 49, 107-119.
HANFREY C., SOMMER S., MAYER M.J., BURTIN D. & MICHAEL A.J. 2001 *Arabidopsis* polyamine biosynthesis: absence of ornithine decarboxylase and the mechanism of arginine decarboxylase activity. Plant J. 27, 551-560.
HARTMANN M.A. 1998 Plant sterols and the membrane environment. Trends Plant Sci. 3, 170-175.
HARTUNG W. & ABOU-MANDOUR A.A. 1996 A beneficial role of abscisic acid for regenerates of *Ruta graveolens* ssp *divaricata* (Tenore) Gams suffering from transplant shock. J. Appl. Bot.-Angewandte Botanik 70, 221-223.
HATANAKA T., SAWABE E., AZUMA T., UCHIDA N. & YASUDA T. 1995 The role of ethylene in somatic embryogenesis from leaf discs of *Coffea canephora*. Plant Sci. 107, 199-204.
HE Z.H., WANG Z.Y., LI J.M., ZHU Q., LAMB C., RONALD P. & CHORY J. 2000 Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. Science 288, 2360-2363.
HEBERLE-BORS E. 1980 Interaction of activated charcoal and iron chelates in anther cultures of *Nicotiana* and *Atropa belladonna*. Z. Pflanzenphysiol. 99, 339-347.
HECK W.W. & PIRES E.G. 1962a Growth of plants fumigated with saturated and unsaturated hydrocarbon gases and their derivatives. Texas Arg. Sta. Misc. Pub., MP-603.
HECK W.W. & PIRES E.G. 1962b Growth of plants fumigated with saturated and unsaturated hydrocarbon gases. Texas Arg. Sta. Misc. Pub., MP-613 3-12.
HEDDEN P. 1999 Regulation of gibberellin biosynthesis. pp. 161-188 in Hooykaas *et al.,* (eds.) 1999 (q.v.).
HEIDE O.M. 1968 Stimulation of adventitious bud formation in *Begonia* leaves by abscisic acid. Nature 219, 960-961.
HEIDE O.M. 1969 Non-reversibility of gibberellin-induced inhibition of regeneration in *Begonia* leaves. Physiol. Plant. 22, 671-679.
HEIMER Y., MIZRAHI Y. & BACHRACH U. 1979 Ornithine decarboxylase activity in rapidly dividing plant cells. FEBS Lett. 104, 146-149.
HEINZMANN U. & SEITZ U. 1977 Synthesis of phenylalanine ammonia-lyase in anthocyanin-containing and anthocyanin-free callus cells of *Daucus carota* L. Planta 135, 63-67.
HEINZMANN U., SEITZ U. & SEITZ U. 1977 Purification and substrate specificities of hydroxycinnamate: CoA-ligase from anthocyanin-containing and anthocyanin-free carrot cells. Planta 135, 313-318.
HENKE R.R., HUGHES K.W., CONSTANTIN M.J., HOLLAENDER A.A. & WILSON C.M. (eds.) 1985 *Tissue Culture in Forestry and Agriculture.* Plenum Press, New York, London.
HIRAGA S., ITO H., SASAKI K., YAMAKAWA H., MITSUHARA I., TOSHIMA H., MATSUI H., HONMA M. & OHASHI Y. 2000 Wound-induced expression of a tobacco peroxidase is not enhanced by ethephon and suppressed by methyl jasmonate and coronatine. Plant Cell Physiol. 41, 165-170.
HISAMATSU T., KOSHIOKA M., KUBOTA S., FUJIME Y., KING R.W. & MANDER L.N. 2000 The role of gibberellin biosynthesis in the control of growth and flowering in *Matthiola incana*. Physiol. Plant. 109, 97-105.
HOOKER T.S. & THORPE T.A. 1998 Effects of fluridone and abscisic acid on lateral root initiation and root elongation of excised tomato root cultures *in vitro*. Plant Cell Tissue Organ Cult. 52, 199-203.
HOOYKAAS P.J.J., HALL M.A. & IBBENGA K.R. (eds.) 1999 *Biochemistry and Molecular Biology of Plant Hormones.* Elsevier Science B.V., Amsterdam
HORN W., SCHLEGEL G. & HAUFT B. 1988a *In vitro* stock plant culture of *Kalanchoe* hybrids. Acta Hortic. 226, 627-630.
HORN W., SCHLEGEL G. & JOHN K. 1988b Micropropagation of roses (*Rosa* hybr.). Acta Hortic. 226, 623-626.
HU Y., BAO F. & LI J. 2000. Promotive effect of brassinosteroids on cell division involves a distinct *CycD3*-induction pathway in *Arabidopsis*. Plant J. 24, 693-701.
HUHTINEN O., HONKANEN J. & SIMOLA L.K. 1982 Ornithine- and putrescine-supported divisions and cell colony formation in leaf protoplasts of alders (*Alnus glutinosa* and *A. incana*). Plant Sci. Lett. 28, 3-9.
HUSSEY G. & STACEY N.J. 1981 *In vitro* propagation of potato (*Solanum tuberosum* L.). Ann. Bot. 48, 787-796.
HUSSEY G. 1977 *In vitro* propagation of some members of Liliaceae, Iridaceae and Amaryyllidaceae. Acta Hortic. 78, 303-309.
HUTCHINSON M.J., KRISHNARAJ S. & SAXENA P.K. 1997 Inhibitory effect of GA_3 on the development of thidiazuron-induced somatic embryogenesis in geranium (*Pelargonium* x *hortorum* Bailey) hypocotyls cultures. Plant Cell Rep. 16, 435-438.
HUXTER T.J., REID D.M. & THORPE T.A. 1979 Ethylene production by tobacco (*Nicotiana tabacum*) callus. Physiol. Plant. 46, 374-380.
HUXTER T.J., THORPE T.A. & REID D.M. 1981 Shoot initiation in light- and dark-grown tobacco callus: the role of ethylene. Physiol. Plant. 53, 319-326.
IKEDA M., OJIMA K. & OHIRA K. 1979 Habituation in suspension-cultured soybean cells to thiamine and its precursors. Plant Cell Physiol. 20, 733-740.
IMASEKI H. 1999 Control of ethylene synthesis and metabolism. pp. 209-245 in Hooykaas *et al.,* (eds.) 1999 (q.v.).
ISHITANI M., MAJUMDER A.L., BORNHOUSER A., MICHALOWSKI C.B., JENSEN R.G. & BOHNERT H.J. 1996 Coordinate transcriptional induction of *myo*-inositol metabolism during environmental stress. Plant J. 9, 537-548.
ISHIZAKI T., KOMAI F., MSUDA K. & MEGUMI C. 2000 Exogenous ethylene enhances formation of embryogenic callus and inhibits embryogenesis in cultures of explants of spinach roots. J. Am. Hortic. Sci. 125, 21-24.
ISIKAWA H. 1974 *In vitro* formation of adventitious buds and roots on the hypocotyls of *Cryptomeria japonica*. Bot. Mag. 87, 73-77.
IWASAKI T. & SHIBAOKA H. 1991 Brassinosteroids act as regulators of tracheary element differentiation in isolated zinnia mezophyll cells. Plant Cell Physiol. 32, 1007-1014.
JACKSON G.V.H., BALL E.A. & ARDITTI J. 1977 Seed germination and seedling proliferation of taro, *Colocasia esculenta* (L.) Schott i*n vitro*. J. Hortic. Sci. 52, 169-171.
JACKSON J.A. & LYNDON R.F. 1988 Cytokinin habituation in juvenile and flowering tobacco. J. Plant Physiol. 132, 575-579.

JACKSON M.B. 1985a Ethylene and responses of plants to soil waterlogging and submergence. Annu. Rev. Plant Physiol. 36, 145-174.

JACKSON M.B. 1985b Ethylene and the responses of plants to excess water in their environment – a review. pp. 241-265 in Roberts J.A. and Tucker J.A. (eds.) *Ethylene and Plant Development*. Butterworths, London.

JACKSON M.B., MANTELL S.H. & BLAKE J. (eds.) 1987 *Advances in the Chemical Manipulation of Plant Tissue Cultures*. Monograph 16, British Plant Growth Regulator Group, Bristol.

JACKSON M.B. 1991 in Davies and Jones (eds.) *Abscisic acid: Physiology & Biochemistry.* BIOS, Oxford 217-226.

JACOBONI A. & STANDARDI A. 1987 Tissue culture of jojoba (*Simmondsia chinensis* Link.). Acta Hortic. 212, 557-560.

JACOBS G., ALLAN P. & BORNMAN C.H. 1969 Tissue culture studies on rose: use of shoot tip explants. I. Auxin: cytokinin effects. II. Cytokinin: gibberellin effects. Agroplantae 1, 179-187.

JACOBS G., ALLAN P. & BORNMAN C.H. 1970 Tissue culture studies on rose: use of shoot tip explants. III. Auxin: gibberellin effects. Agroplantae 2, 45-49.

JAMES D.J. & WAKERELL I.J. 1982 The control of rhizogenesis *in vitro* in difficult-to-root apple rootstocks. pp. 187-188 in Fujiwara (ed.) 1982 (q.v.).

JAMES D.J., PASSEY A.J. & RUGINI E. 1988 Factors affecting high frequency plant regeneration from apple leaf tissue cultured *in vitro*. J. Plant Physiol. 132, 148-154.

JARRET R.L. & HASEGAWA P.M. 1981 An analysis of the effect of gibberellic acid on adventitious shoot formation and development from tuber discs of potato. Env. Exp. Bot. 21, 436.

JARVIS B.C., SHANNON P.R.M. & YASMIN S. 1983 Involvement of polyamines with adventitious root development in stem cuttings of mung bean. Plant Cell Physiol. 24, 677-683.

JARVIS B.C., YASMIN S. & COLEMAN M. 1985 RNA and protein metabolism during root formation in stem cuttings of *Phaseolus aureus* cultivar Berkin. Plant Physiol. 64, 53-59.

JELASKA S. 1987 Micropropagation of juvenile *Calocedrus decurrens*. Acta Hortic. 212, 449-456.

JERIE P.H. & HALL M.A. 1978 The identification of ethylene oxide as a major metabolite of ethylene in *Vicia faba* L. Proc. Roy. Soc. Lond. 200,87-94.

JIMENEZ V.M. & BANGERTH F. 2000 Relationship between endogenous hormone levels of grapevine callus cultures and their morphogenetic behaviour. Vitis 39, 151-157.

JIMENEZ V.M. & BANGERTH F. 2001 Endogenous hormone levels in explants and in embryogenic and non-embryogenic cultures of carrot. Physiol. Plant. 111, 389-395.

JOHANSSON L 1983 Effects of activated charcoal in anther cultures. Phdiol. Plant. 59, 397-403.

JOHANSSON L., ANDERSSON B. & ERIKSSON T. 1982 Improvement of anther culture: activated charcoal bound in agar medium in combination with liquid medium and elevated CO_2 concentration. Physiol. Plant. 54, 24-30.

JOHNSON P.R. & ECKER J.R. 1998 The ethylene gas signal transduction pathway: a molecular perspective. Annu. Rev. Genet. 32, 227-254.

JOHRI B.M. & BHOJWANI S.S. 1977 Triploid plants through endosperm culture. pp. 398-411 in Reinert and Bajaj (eds.) 1977 (q.v.).

JONA R. & VIGLIOCCO R. 1985 Axillary bud culture of peach. Acta Hortic. 173, 223-228.

JONES O.P., HOPGOOD M.E. & O'FARRELL D. 1977 Propagation *in vitro* of M26 apple rootstocks. J. Hort. Sci. 52, 235-238.

JONES O.P., PONTIKIS C.A. & BEECH M.G. 1979 Propagation *in vitro* of five apple scion cultivars. J. Hort. Sci. 54, 155-158.

KAKKAR R.K. & RAI V.R. 1987 Effects of spermine and IAA on carbohydrate metabolism during rhizogenesis in *Phaseolus vulgaris*. Ind. J. Exp. Biol. 25, 476-478.

KAMADA H. & HARADA H. 1981 Changes in the endogenous levels and effects of abscisic acid during somatic embryogenesis of *Daucas carota* L. Plant Cell Physiol. 22, 1423-1429.

KARTHA K.K., CHAMPOUX S., GAMBORG O.L., & PAHL K. 1977 *In vitro* propagation of tomato by shoot apical meristem culture. J. Am. Soc. Hortic. Sci. 102, 346-349.

KARTHA K.K., GAMBORG O.L., CONSTABEL F. & KAO K.N. 1974a Fusion of rape seed and soyabean protoplasts and subsequent division of heterokaryocytes. Can J. Bot. 52, 2435-2436.

KARTHA K.K., GAMBORG O.L., CONSTABEL F. & SHYLUK J.P. 1974b Regeneration of cassava plants from apical meristems. Plant Sci. Lett. 2, 107-113.

KATO Y. & HONGO M. 1974 Regulation of bud and root formation in excised leaf segments of *Heloniopsis orientalis* (Liliaceae). Phytomorph. 24, 273-279.

KAUR-SAWHNEY R., TIBURCIO A.F. & GALSTON A.W. 1988 Spermidine and flower-bud differentiation in thin-layer explants of tobacco. Planta 173, 282-284.

KEBEBEW A., GAJ M.D. & MALUSZYNSKI M. 1998 Somatic embrogenesis and plant regeneration in callus culture of tef *Eragrostis tef* (Zucc) Trotter. Plant Cell Rep. 18, 154-158.

KEFU Z., MUNNS R. & KING R.W. 1991 Abscisic acid levels in NaCl-treated barley, cotton and saltbush. Aust. J. Plant Physiol 18, 17-24.

KELLER R., BREARLEY C.A., TRETHEWEY R.N. & MULLER-ROBER B. 1998 Reduced inositol content and altered morphology in transgenic potato plants inhibited for 1D-*myo*-inositol 3-phosphate synthase. Plant J. 16, 403-410.

KENDE H., KONZE J.R. & BOLLER T. 1980 Enzymes of ethylene biosynthesis. pp. 230-238 in Skoog (ed.) 1980 (q.v.).

KERBAUY G.B., PETERS J.A. & HELL K.G. 1986 Cytokinin autotrophy and differentiation in tissue cultures of haploid *Nicotiana tabacum* L. Plant Sci. 45, 125-132.

KERNS H.R. & MEYER M.M.Jr. 1986 Tissue culture propagation of *Acer* x *freemanii* using thidiazuron to stimulate shoot tip proliferation. HortScience 21, 1209-1210.

KEVERS C., BOYER N., COURDUROUX J.-C. & GASPAR TH. 1992 The influence of ethylene on proliferation and growth of rose shoot cultures. Plant Cell Tissue Organ Cult. 28, 175-181.

KEVERS C., FILALI M., PETIT-PALY G., HAGEGE D., RIDEAU M. & GASPAR TH. 1996 Habituation of plant cells does not mean insensitivity to plant growth regulators. In Vitro Cell. Dev. Biol.-Plant 32, 204-209.

KEVERS C., GASPAR TH., PENEL C. & GREPPIN H. 1985 Auxin-polyamine interaction with the calcium-controlled secretion of peroxidase by sugar beet cells. Plant Cell Rep. 4, 120-122.

KHALAFALLA M.M. & HATTORI K. 2000 Ethylene inhibitors enhance *in vitro* root formation on faba bean shoots regenerated on medium containing thidiazuron. Plant Growth Regul. 32, 59-63.

KHAN A.J & MINOCHA S.C. 1991 Polyamines and somatic embryogenesis in carrot. II. The effects of cyclohexyl-ammonium phosphate. J. Plant Physiol. 137, 446-452.

KIEBER J.J., ROTHENBERG M., ROMAN G., FELDMAN K.A. & ECKER J.R. 1993 CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. Cell 72, 427-441.

KIM Y.W., YOUN Y., NOH E.R. & KIM J.C. 1998 Somatic embryogenesis and plant regeneration from immature zygotic embryos of Japanese larch (*Larix leptolepis*). Plant Cell Tissue Organ Cult. 55, 95-101.

KOCHBA J., BUTTON J., SPIEGEL-ROY P., BORNMAN C.H. & KOCHBA M. 1974 Stimulation of rooting of citrus embryoids by gibberellic acid and adenine sulphate. Ann. Bot. 38, 795-802.

KOCHBA J., SPIEGEL-ROY P. & SAFRAN H. 1972 Adventive plants from ovules and nucelli in *Citrus*. Planta 106, 237-245.

KOCHBA J., SPIEGEL-ROY P., NEUMANN H. & SAAD S. 1978 Stimulation of embryogenesis in *Citrus* ovular callus by ABA, ethephon, CCC and Alar and its suppression by GA_3. Z. Pflanzenphysiol. 89, 427-432.

KOCHHAR T.S. 1980 Effect of abscisic acid and auxins on the growth of tobacco callus. Z. Pflanzenphysiol. 97, 1-4.

KOETJE D.S., KONONOWICZ H. & HODGES T.K. 1993 Polyamine metabolism associated with growth and embryo-genic potential of rice. J. Plant Physiol. 141, 215-221.

KOHLENBACH H. & WERNICKE W. 1978 Investigations of the inhibitory effect of agar and the function of active carbon in anther culture. Z. Pflanzenphysiol. 86, 463-472.

KOMAI F., OKUSE I., SAGA K. & HARADA T. 1996 Improvement on the efficiency of somatic embryogenesis from spinach root tissues by applying various sugars. J. Jap. Soc. Hortic. Sci. 65, 67-72.

KOMAMINE A., MATSUMOTO M., TSUKAHARA M., FUJIWARA A., KAWAHARA R., ITO M., SMITH J., NOMURA K. & FUJIMURA T. 1990 Mechanism of somatic embryogenesis in cell cultures – physiology, biochemistry and molecular biology. pp. 307-313 in Nijkamp *et al.,* (eds.) 1990 (q.v.).

KONAR R.N. & KONAR A. 1973 Interaction of CCC and gibberellic acid in the morphogenesis of *Ranunculus sceleratus* tissue *in vitro*. Phytomorph. 23, 105-109.

KROGSTRUP P., ERIKSEN E.N., MØLLER J.D. & ROULUND H. 1988 Somatic embryogenesis in Sitka spruce (*Picea sitchensis* Bong. Carr.). Plant Cell Rep. 7, 594-597.

KU H.S., SUGE H., RAPPAPORT L. & PRATT A.K. 1970 Stimulation of rice coleoptile growth by ethylene. Planta 90, 333-339.

KUKREJA A.K. & MATHUR A.K. 1985 Tissue culture studies in *Duboisia myoporoides*: 1. Plant regeneration and clonal propagation by stem node cultures. Planta Med. 51, 93-96.

KULPA J.M., GALSKY A.G., LIPETZ P. & STEPHENS R. 1985 Polyamines and crown gall tumor growth. Plant Cell Rep. 4, 81-83.

KUMAR P.P., REID D.M. & THORPE T.A. 1986 Ethylene and carbon dioxide accumulation and morphogenesis in excised cotyledons of *Pinus radiata*. p. 11 in Abstr. VI Congr. Plant Tissue & Cell Culture, Minneapolis, Minn.

KUMAR P.P., REID D.M. & THORPE T.A. 1987 The role of ethylene and carbon dioxide in differentiation of shoot buds in excised cotyledons of *Pinus radiata in vitro*. Physiol. Plant. 69, 244-252.

LAKSHMI SITA G., RAGHAVA RAM N.V. & VAIDYANATHAN C.S. 1979 Differentiation of embryoids and plantlets from shoot callus of sandalwood. Plant Sci. Lett. 15, 265-270.

LALORAYA M.M., SRIVASTAN H.N. & GURUPRASAD K.N. 1976 Recovery of gibberellic acid inhibition of betacyanin biosynthesis by pigment precursors. Planta 128, 275-276.

LAMBE P., MUTAMBEL H.S.N., FOUCHE J.G., DELTOUR R., FOIDART J.M. & GASPAR TH. 1997 DNA methylation as a key process in regulation of organogenic totipotency and plant neoplastic progression? In Vitro Cell. Dev. Biol.-Plant 33, 155-162.

LANE W.D. 1978 Regeneration of apple plants from shoot meristem tips. Plant Sci. Lett. 13, 281-285.

LANE W.D. 1979 Influence of growth regulators on root and shoot initiation from flax meristem tips and hypocotyls *in vitro*. Physiol. Plant. 45, 260-264.

LANGOWSKA T. 1980 Effect of activated carbon and brown coal suspensions for growth and development of *Scenedesmus obliquus* (SC. 449). Pol. Arch. Hydrobiol. 27, 125-136.

LARUE T.A.G. & GAMBORG O.L. 1971 Ethylene production by plant cell cultures. Variations in production during growing cycle and in different plant species. Plant Physiol. 48, 394-398.

LATIES G.G. 1962 Controlling influence of thickness on development and type of respiratory activity in potato slices. Plant Physiol. 37, 679-690.

LAU O.-L. & YANG S.F. 1973 Mechanism of a synergistic effect of kinetin on auxin-induced ethylene production. Plant Physiol. 51, 1011-1014.

LAU O.-L. & YANG S.F. 1976 Stimulation of ethylene production in the mung bean hypocotyls by cupric ion, calcium ion, and kinetin. Plant Physiol. 57, 88-92.

LE GUYADER H. 1987 Ethylène et différenciation de racines par des cultures de couches cellulaires minces de tabac. Compt. Rend. Acad. Sci. Paris 305, 591-594.

LE RUDULIER D. & GOAS G. 1975 Effect of ammonium and potassium ions on the accumulation of putrescine in young decotylized *Soja hispida* plants. Physiol. Veg. 13, 125-136.

LEDILY F., BILLARD J.P., HUAULT C., KEVERS C. & GASPAR TH. 1993 Fully habituated sugarbeet callus - under permanent stress. In Vitro Cell. Dev. Biol.-Plant 29P, 149-154.

LEE C.W.T. & SHULER M.L. 1991 Different shake flask closures alter gas-phase composition and ajmalicine production in *Catharanthus roseus* cell suspensions. Biotechnol. Tech. 5, 173-178.

LEE T.M. & CHU. C. 1992 Ethylene-induced polyamine accumulation in rice (*Oryza sativa* L.) coleoptiles. Plant Physiol. 100, 238-245.

LEE T.M., LUR H.S., LIN Y.H. & CHU C. 1996 Physiological and biochemical changes related to methyl jasmonate-induced chilling tolerance of rice (*Oryza sativa* L.) seedlings. Plant Cell Environ. 19, 65-74.

LEMBI C.A., MORRÉ D.J., THOMSON K.-St. & HERTEL R. 1971 N-1-Naphthylphthalamic acid-binding activity of a plasma membrane-rich fraction from maize coleoptiles. Planta 99, 37-45.

LEON J., ROJO E. & SANCHEZ-SERRANO J.J. 2001 Wound signalling in plants. J. Exp. Bot. 52, 1-9.

LEPAGE-DEGIVRY M.-T. 1973 Étude en culture *in vitro* de la dormance embryonnaire de *Taxus baccata* L. Biol. Plant. 15, 264-269.

LESHEM Y.Y., WILLS R.B.H. & KU V.V.V. 1998 Evidence for the function of the free radical gas - nitric oxide ($NO^{\cdot}$) - as an endogenous maturation and senescence regulating factor in higher plants. Plant Physiol. Biochem. 36, 825-833.

LEVY D., SEABROOK J.E.A. & COLEMAN S. 1993 Enhancement of tuberization of axillary shoot buds of potato (*Solanum tuberosum* L.) cultivars cultured *in vitro*. J. Exp. Bot. 44, 381-386.

LI H.C., RICE E.L., RONHRBAUGH L.M. & WENDER S.H. 1970 Effects of abscisic acid on phenolic content and lignin biosynthesis in tobacco tissue culture. Physiol. Plant. 23, 928-936.

LI J. & CHORY J. 1997 A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90, 929-938.

LI J., NAGPAL P., VITART V., McMORRIS T.C. & Chory J. 1996 A role for brassinosteroids in light-dependent development of *Arabidopsis*. Science 272, 398-401.

LINSMAIER E.M. & SKOOG F. 1965 Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18, 100-127.

LITZ R.E. 1987 Application of tissue culture to tropical fruits. pp. 407-418 in Green C.E, Sommers D.A., Hackett W.P. & Biesboer D.D. (eds.) 1987 *Plant Tissue and Cell Culture*. Alan R. Liss, Inc., New York.

LU C.Y. & VASIL I.K. 1981 Somatic embryogenesis and plant regeneration from leaf tissues of *Panicum maximum* Jacq. Theor. Appl. Genet. 59, 275-280.

LU C.Y. & VASIL I.K. 1982 Somatic embrogenesis and plant regeneration in tissue cultures of *Panicum maximum* Jacq. Am. J. Bot. 69, 77-81.

LU C.Y., VASIL I.K. & OZIAS-AKINS P. 1982 Somatic embryogenesis in *Zea mays*. Theor. Appl. Genet. 62, 109-112.

MACKENZIE I.A. & STREET H.E. 1970 Studies on the growth in culture of plant cells. VIII. Production of ethylene by suspension cultures of *Acer pseudoplatanus* L. J. Exp. Bot. 21, 824-834.

MAENE L. & DEBERGH P. 1985 Liquid medium additions to establish tissue cultures to improve elongation and rooting *in vitro*. Plant Cell Tissue Organ Cult. 5 23-33.

MALMBERG R.L., WATSON M.B., GALLOWAY G.L. & YU W. 1998 Molecular genetic analyses of plant polyamines. Crit. Rev. Plant Sci. 17, 199-224.

MARANI F. & PISI A. 1977 Meristem-tip culture and vegetative propagation in potato. Acta Hortic. 78, 415-422.

MARGARA J. 1969 Étude des facteurs de la néoformation de bourgeons et culture *in vitro* chez le chou-fleur (*Brassica oleracea* L. var. *botrytis*). Ann Physiol. Veg. 11, 95-112.

MARTIN C., KUNESCH G., MARTIN-TANGUY J., NEGREL J., PAYNOT M. & CARRÉ M. 1985 Effect of cinnamoyl putrescines on *in vitro* cell multiplication and differentiation of tobacco explants. Plant Cell Rep. 4, 158-160.

MARTIN G.C. 1987 Apical dominance. HortScience 22, 824-833.

MARTINEZ-GARCIA J.F. & GARCIA-MARTINEZ J.L. 1992 Effect of the growth retardant Lab-198 999, an acylcyclo-hexanedione compound, on epicotyl elongation and metabol-ism of gibberellin-A1 and gibberellin-A20 in cowpea. Planta 188, 245-251.

MARTIN-TANGUY J., MARTIN C., PAYNOT M. & ROSSIN N. 1988 Effect of hormone treatment on growth bud formation and free amine and hydroxycinamoil putrescine levels in leaf explant of *Nicotiana tabacum* cultivated *in vitro*. Plant Physiol. 88, 600-604.

MATA C.G. & LAMATTINA L. 2001 Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiol. 126, 1196-1204.

MATTOO A.K. & WHITE B. 1991 Regulation of ethylene biosynthesis. pp.21-42 in Mattoo A.K. and Suttle J.C. (eds.) *The Plant Hormone Ethylene*, CRC Press, Boca Raton, Florida

MAUSETH J.D. & HALPERIN W. 1975 Hormonal control of organogenesis in *Opuntia polyacantha* (Cactaceae). Am. J. Bot. 62, 869-877.

McCULLOCH S.M. 1988 Commercial micropropagation of *Kalmia*. Comb. Proc. Int. plant Prop. Soc. 1987 37, 107-109.

McGLASSON W.B., WADE N.L. & ADATO I. 1978 Phytohormones and fruit ripening. pp. 447-493 in Letham D.S., Goodwin P.B. and Higgins T.J.V. (eds.) 1978 *Phytohormones and Related Compounds: A Compre-hensive Treatise*. Elsevier, North-Holland Biomedical Press, Amsterdam.

MCGURL B., OROZCOCARDENAS M., PEARCE G. & RYAN C.A. 1994 Over-expression of the prosystemin gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase inhibitor synthesis. Proc. Natl. Acad. Sci. USA 91, 9799-9802.

McKEON T.A., FERNANDEZ-MACULET J.C. & YANG S.F. 1995 Biosynthesis and metabolism of ethylene. pp. 118-139 in Davies P.J. (ed.) 1995 *Plant Hormones: Physiology, Biochemistry and Molecular Biology*. Kluwer, Dordrecht

MEHRA A. & MEHRA P.N. 1972 Differentiation in callus cultures of *Mesembryanthemum floribundum*. Phytomorph. 22, 171-176.

MEIJER E.G.M & BROWN D.C.W. 1988 Inhibition of somatic embryogenesis in tissue cultures of *Medicago sativa* by aminoethoxyvinylglycine, amino-oxyacetic acid, 2,4-dinitro-phenol and salicylic acid at concentrations which do not inhibit ethylene biosynthesis and growth. J. Exp. Bot. 39, 263-270.

MEINS F. Jr. 1974 Mechanisms underlying the persistence of tumour autonomy in crown gall disease. pp. 233-264 in Street H.E. (ed.) 1974 *Plant Tissue and Plant Science.* Academic Press. London, New York, San Francisco.

MEINS F. Jr. & BINNS A.N. 1978 Epigenetic clonal variation in the requirement of plant cells for cytokinins. Symp. Soc. Dev. Biol. 36, 188-201.

MEINS F. Jr. & FOSTER R. 1986 A cytokinin mutant derived from cultured tobacco cells. Dev. Genet. 7, 159-165.

MEINS F. Jr. & LUTZ J. 1980 Epigenetic changes in tobacco cell culture: studies of cytokinin habituation. pp. 220-236 in Rubenstein L., Gengenbach B. Phillips, R.L and Green C.E. (eds.) *1980 Genetic Improvment of Crop Plants – Emergent Techniques.* Univ. Minnesota Press, Minneapolis.

MEINS F. Jr. 1987 Hormones and the molecular basis of determination in plants. pp. 19-28 in Jackson *et al.,* (eds.) 1987 (q.v.).

MEINS F. Jr., FOSTER R. & LUTZ J.D. 1983 Evidence for a Mendelian factor controlling the cytokinin requirement of cultured tobacco cells. Dev. Genet. 4, 129-141.

MELÉ E., MESSEGUER J. & CAMPRUBI P. 1982 Effect of ethylene on carnation explants grown in sealed vessels. pp. 69-70 in Fujiwara (ed.) 1982 (q.v.).

MELLOR F.C. & STACE-SMITH R. 1969 Development of excised potato buds in nutrient medium. Can. J. Bot. 47, 1617-1621.

MENSUALISODI A., PANIZZA M., SERRA G. & TOGNONI F. 1993 Involvement of activated charcoal in the modulation of abiotic and biotic ethylene levels in tissue cultures. Sci. Hortic. 54, 49-57.

MIKLASHEVICHS E., ROHRIG H., SCHELL J. & SCHMIDT J. 2001 Perception and signal transduction of rhizobial NOD factors. Crit. Rev. Plant Sci. 20, 373-394.

MILBORROW B.V. 1974 The chemistry and physiology of abscisic acid. Annu. Rev. Plant Physiol. 25, 259-307.

MILLER A.R. & ROBERTS L.W. 1982 Regulation of tracheary element differentiation by exogenous L-methionine in callus of soya bean cultivars. Ann. Bot. 50, 111-116.

MILLER A.R., CRAWFORD D.L. & ROBERTS L.W. 1985 Lignification and xylogenesis in *Latuca* pith explants cultured *in vitro* in the presence of auxin and cytokinin: a role for endogenous ethylene. J. Exp. Bot. 36, 110-118.

MINOCHA S.C. & NISSEN P. 1985 Uptake of abscisic acid in tuber slices of Jerusalem artichoke and potato. J. Plant Physiol. 120, 313-320.

MIRJALILI N. & LINDEN J.C. 1995 Gas-phase composition effects on suspension cultures of *Taxus cuspidata*. Biotechnol. Bioeng. 48, 123-132.

MIZUKAMI H., TOMITA K., OHASHI H. & HIRAOKA N. 1988 Anthocyanin production in callus cultures of roselli (*Hibiscus sabdariffa* L.). Plant Cell Rep. 7, 553-556.

MOHEN D., SHINSHI H,M FELIX G. & MEINS F. 1985 Hormonal regulation of β-1,3-glucanase messenger RNA levels in cultured tobacco tissues. EMBO J. 4, 1631-1635.

MOK M.C. & MOK D.W.S. 1979 Cytokinin independence in tissue cultures of *Phaseolus*: a genetic trait. In Vitro 15, 190.

MOK M.C., CAPELLE S.C., KIRCHNER S.C., MOK D.W.S. & ARMSTRONG D.J. 1982 The effects of N-phenyl-N´-1,2,3-thiadiazol-5-ylurea (thidiazuron) on cytokinin autonomy and the metabolism of ^{14}C-N6-(Δ2-isopentenyl)adenosine (^{14}C-6Ado) in callus cultures of *Phaseolus lunatus* L. In Vitro 18, 275-275.
MONCOUSIN Ch. & GASPAR TH. 1983 Peroxidase as a marker for rooting improvement of *Cynara scolymus* L. cultured *in vitro*. Biochem. Physiol. Pflanz. 178, 263-271.
MOORE G.M. 1985 Mechanisms of hormone action in plants. Comb. Proc. Int. Plant Prop. Soc. 1984. 34, 79-90.
MOORE R. & SMITH J.D. 1984 Growth, graviresponsiveness and abscisic acid content of *Zea mays* seedlings treated with fluridone. Planta 162, 342-344.
MOREL G., MARTIN C. & MÜLLER J.F. 1968 La guérison des pomme de terre atteintes maladies à virus. Ann. Physiol. Veg. 10, 113-139.
MOSHKOV I.E, NOVIKOVA G.V., MUR L.A.J., SMITH A.R. & HALL M.A. 2003a Ethylene rapidly up-regulates the activities of both monomeric GTP-Binding proteins and protein kinase(s) in epicotyls of pea. Plant Physiol. 131, 1718-1726
MOSHKOV I.E, NOVIKOVA G.V., MUR L.A.J., SMITH A.R. & HALL M.A. 2003b Ethylene regulates monomeric GTP-binding protein gene expression and activity in *Arabidopsis thaliana* Plant Physiol. 131, 1705-1717
MUHITCH M.J., EDWARDS L.A. & FLETCHER J.S. 1983 Influence of diamines and polyamines on the senescence of plant suspension cultures. Plant Cell Rep. 2, 82-84.
MUKUND S., ROBACKER C. & SHEWFELT R. 1988 Callus and suspension cultures of tomato fruits for postharvest studies. HortScience 23, 754.
MULLINS M.G. & SRINIVASAN C. 1976 Somatic embryos and plantlets from an acient clone of the grapevine (cv. Cabernet-Sauvignon) by apomixes *in vitro*. J. Exp. Bot. 27, 1022-1030.
MURASHIGE T. 1961 Supression of shoot formation in cultured tobacco cells by gibberellic acid. Science. 134, 280.
MURASHIGE T. 1964 Analysis of the inhibition of organ formation in tobacco tissue culture by gibberellin. Physiol. Plant. 17, 636-643.
MUTAFTSCHIEV S., COUSSON A. & TRAN THANH VAN K. 1987 Modulation of cell growth and differentiation by pH and oligosaccharides. pp. 29-42 in Jackson *et al.,* (eds.) 1987 (q.v.).
NAIRN B.J. 1988 Significance of gelling agents in a production tissue culture laboratory. Comb. Roc. Int. Plant Prop. Soc. 37, 200-205.
NANDA K.K., ANAND V.K. & CHIBBAR R.N. 1972 The promotive effect of gibberellic acid on the production of adventitious roots on stem cuttings of *Ipomoea fistulata*. Planta 105, 360-363.
NARVAEZVASQUEZ J., OROZCOCARDENAS M.L. & RYAN C.A. 1994 A sulfhydryl reagent modulates systemic signaling for wound-induced and systemin-induced proteinase inhibitor synthesis. Plant Physiol. 105, 725-730.
NELSON D.E., RAMMESMAYER G. & BOHNERT H.J. 1998 Regulation of cell-specific inositol metabolism and transport in plant salinity tolerance. Plant Cell 10, 753-764.
NEMETH G. 1979 Benzyladenine-stimulated rooting in fruit-tree rootstocks cultured *in vitro*. Z. Pflanzenphysiol. 95, 389-396.
NEWBURY H.J. 1986 Multiplication of *Antirrhinum majus* L. by shoot-tip culture. Plant Cell Tissue Organ Cult. 7, 39-42.
NIJKAMP H.J.J., VAN DER PLAS I.H.W. & VAN AARTRIJK (eds.) 1990 *Progress in Plant Cellular and Molecular Biology.* Proc. VIIth Int. Cong. on Plant Tissue and Cell Culture. Amsterdam, The Netherlands. 24-29 June 1990. Kluwer Academic Publishers, Dortrecht, Netherlands.
NISSEN S.J. & SUTTER E.G. 1988 Stability of IAA and IBA in nutrient medium after autoclaving and after storage under various environmental conditions. HortScience 23, 758.
NISSEN S.J. & SUTTER E.G. 1990 Stability of IAA and IBA in nutrient medium to several tissue culture procedures. HortScience 25, 800-802.
NOH E.W., MINOCHA S.C. & RIEMENSCHNEIDER D.E. 1988 Adventitious shoot formation from embryonic explants of red pine (*Pinus resinosa*). Physiol. Plant. 74, 119-124.
NOMA M., HUBER J., ERNST D. & PHARIS R. 1982 Quantitation of gibberellins and the metabolism of ^{3}H-gibberellin A1 during somatic embryogenesis in carrot and aniseed cell cultures. Planta 155, 369-376.
NOMURA T., KITASAKA Y., TAKATSUTO S., REID J.B., FUKAMI M. & YOKOTA T. 1999 Brassinosteroid/sterol synthesis and plant growth as affected by *Ika* and *Ikb* mutations of pea. Plant Physiol. 119, 1517-1526.
NOVÁK F.J., ZADINA J., HORACKOVA V. & MASKOVA L. 1980 The effect of growth regulators on meristem tip development and *in vitro* multiplication of *Solanum tuberosum* L.plants. Potato Res. 23, 155-160.
NOVIKOVA G.V., MOSHKOV I.E., SMITH A.R. & HALL M.A. 1997 The effect of ethylene on GTP binding in extracts from pea seedlings. Planta 201, 1-8.
NOVIKOVA G.V., MOSHKOV I.E., SMITH A.R. & HALL M.A. 2000 The effect of ethylene on MAPKinase-like activity in *Arabidopsis thaliana*. FEBS Lett. 474, 29-32.
NOVIKOVA G.V., MOSHKOV I.E., SMITH A.R., KULAEVA O.N. & HALL M.A. 1999 The effect of ethylene and cytokinin on guanosine 5´-triphosphate binding and protein phosphorylation in leaves of *Arabidopsis thaliana*. Planta 208, 239-246.
NÚÑEZ M., SIQUEIRA W.J., HERNÁNDEZ M., ZULLO M.A.T., ROBAINA C. & COLL, F. 2004. Effect of spirostane analogues of brassinosteroids on callus formation and plant regeneration in lettuce (*Lactuca sativa*). Plant Cell Tissue and Organ Cult. 78, 97-99.
NWANKWO B.A. & KRIKORIAN A.D. 1983 Morphogenetic potential of embryo and seedling-derived callus of *Elaeis guineensis* Jacq. var. *pisifera* Becc. Ann. Bot. 51, 459-466.
OH M.H. & CLOUSE S.D. 1998 Brassinolide affects the rate of cell division in isolated leaf protoplasts of *Petunia hybrida*. Plant Cell Rep. 17, 921-924.
OSHMARINA V.I., SHEVYAKOVA N.I. & SHAMINA Z.B. 1982 Dynamics of free amino acids and amides in *Nicotiana sylvestris* cell cultures at different concentrations of putrescine in the medium. Sov. Plant Physiol. 29, 477-481.
OWEN H.R., WENGERD D. & MILLER A.R. 1991 Culture medium pH influenced by basal medium, carbohydrate source, gelling agent, activated charcoal, and medium storage method. Plant Cell Rep. 10, 583-586.
PALAVAN N., GOREN R. & GALSTON A.W. 1984 Effects of some growth regulators on polyamine biosynthetic enzymes in etiolated pea seedlings. Plant Cell Physiol. 25, 541-546.
PAL BAIS H. & RAVISHANKAR G.A. 2002 Role of polyamines in the ontogeny of plants and their biotechnological applications. Plant Cell Tissue and Organ Cult. 69, 1-34.
PAN M.J. & VAN STADEN J. 1998 The use of charcoal in *in vitro* culture. Plant Growth Regul. 26, 155-163.
PANAIA M., SENARATNA T., BUNN E., DIXON K.W. & SIVASITHAMPARAM K. 2000 Micropropagation of the critically endangered Western Australian species, *Symonanthus bancroftii* (F. Muell.) L. Haegi (Solanaceae). Plant Cell Tissue Organ Cult. 63, 23-29.
PANDEY K.N. & SABHARWAL P.S. 1979 *In vitro* neoplastic transformation of plant callus tissue by γ-radiation. Mutation Res. 62, 459-465.

PARANJOTHY K. & ROHANI O. 1982 *In vitro* propagation of oil palm. pp. 747-749 in Fujiwara (ed.) 1982 (q.v.).

PATEL B.N., CATTELL K.J. & HOCKING T.J. 1986 Uptake of abscisic acid by isolated mesophyll protoplast from *Vicia faba*. Plant Sci. 45, 195-199.

PATTNAIK S.K. & CHAND P.K. 1997 Rapid clonal propagation of three mulberries, *Morus cathayana* Hemsl, M. ihou Koiz and *M. serrata* Roxb, through *in vitro* culture of apical shoot buds and nodal explants from mature trees. Plant Cell Rep. 16, 503-508.

PEDROSO M.C., MAGALHAES J.R. & DURZAN D. 2000 A nitric oxide burst precedes apoptosis in angiosperm and gymnosperm callus cells and foliar tissues. J. Exp. Bot. 51, 1027-1036

PENNAZIO S. & RADOLFI P. 1973 Factors affecting the culture *in vitro* of potato meristem tips. Potato Res. **16**, 20-29.

PERL A., AVIV D. & GALUN E. 1988 Ethylene and *in vitro* culture of potato: suppression of ethylene generation vastly improves protoplast yield, plating efficiency and transient expression of an alien gene. Plant Cell Rep. 7, 403-406.

PHILLIPS G.C. & COLLINS G.B. 1981 Induction and development of somatic embryous from cell suspension cultures of soybean. Plant Cell Tissue Organ Cult. 1, 123-129.

PHILLIPS R. & DODDS J.H. 1977 Rapid differentiation of tracheary elements in cultured explants of Jerusalem artichoke. Planta 135, 207-212.

PHILLIPS R. 1987 Effects of sequential exposure to auxin and cytokinin on xylogenesis in cultured explants of Jerusalem artichoke (*Helianthus tuberosus* L.). Ann. Bot. 59, 245-250.

PIIRONEN V., LINDSAY D.G., MIETTINEN T.A., TOIVO J. & LAMPI A.M. 2000 Plant sterols: biosynthesis, biological function and their importance to human nutrition. J. Sci. Food Agric. 80, 939-966.

PILLAI S.K. & HILDERBRANDT A.C. 1969 Induced differentiation of geranium plants from undifferentiated callus *in vitro*. Am. J. Bot. 56, 52-58.

PITTET H. & MONCOUSIN C. 1982 La micropropagation *in vitro* du rosier. Rev. Hort. Suisse 55, 67-69.

PODER D., OMARZAD O. & HAGEGE D. 1998 Comparative study of respiratory metabolism in three types of sugarbeet calli, a normal and two habituated ones (organogenic and non-organogenic). Comptes Rendus Acad. Sci. Ser. III-Sci. Vie-Life Sci. 321, 479-483.

PUA E.-C., CHONG C. & ROUSSELLE G.L. 1983 *In vitro* propagation of Ottowa 3 apple rootstock. Can. J. Plant Sci. 63, 183-188.

PULLMAN G.S., ZHANG Y. & PHAN B.H. 2003. Brassinolide improves embryogenic tissue initiation in conifers and rice. Plant Cell Rep. 22, 96-104.

PURNHAUSER L., MEDGYESY P., CZAKO M., DIX P.J. & MARTON L. 1987 Stimulation of shoot regeneration in *Triticum aestivum* and *Nicotiana plumbaginifolia* Viv. tissue cultures using the ethylene inhibitor $AgNO_3$. Plant Cell Rep. 6, 1-4.

PUSCHMANN R., KE D. & ROMANI R. 1985 Ethylene production by suspension-cultured pear fruit cells as related to their senescence. Plant Physiol. 79, 973-976.

PUTZ C. 1971 Obtention de framboisiers (var. Bios blanc) sans virus par la technique des cultures de méristèmes. Phytopath. 3, 493-501.

PYTHOUD F., BUCHALA A.J. & SCHMID A. 1986 Adventitious root formation in green cuttings of *Populus tremula* L., characterization of the effect ov Vit. D3 and indolylbutyric acid. Physiol. Plant. 68, 93-99.

QUAK F. 1977 Meristem culture and virus-free plants. pp. 598-615 in Reinert and Bajaj (eds.) 1977 (q.v.).

RADEMACHER W. 2000 Growth retardants: effects on gibberellin biosynthesis and other metabolic pathways. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 501-531.

RADIN J.W. & LOOMIS R.S. 1969 Ethylene and carbon dioxide in the growth and development of cultured radish roots. Plant Physiol. 44, 1584-1589.

RAHBAR K. & CHOPRA R.N. 1982 Effect of liquid medium, activated charcoal and pH on the onset of reproductive phase in the moss *Bartramidula bartramioides*. Z. Pflanzenphysiol. 106, 185-189.

RAJASEKARAN K., HEIN M.B. & VASIL I.K. 1987 Endogenous abscisic acid and indole-3-acetic acid and somatic embryogenesis in cultured leaf explants of *Pennisetum purpureum* Schum. Effects *in vivo* of glyphosate, fluridone and paclobutrazol. Plant Physiol. 84, 47-51.

RANGA SWAMY N.S. 1959 Morphogenetic response of *Citrus* ovules to growth adjuvants in culture. Nature 183, 735-736.

RANGA SWAMY N.S. 1961 Experimental studies on female reproductive structures of *Citrus microcarpa* Bunge. Phytomorph. 11, 109-127.

RAO P.S. & NARAYANASWAMY S. 1968 Induced morphogenesis in tissue cultures of *Solanum xanthocarpum*. Planta 81, 372-375.

RAO U., NARANG V. & RAO R. 1987 Plant regeneration from somatic embryos of bamboo and their transplantation to soil. pp. 101-107 in Ducaté G., Jacob M. & Simeon A. (eds.) 1987 *Plant Micropropagation in Horticultural Industries. Preparation, Hardening and Acclimatisation Processes*. Belgian Plant Tissue Culture Group, Symposium Florizel 87: Arlon, Belgium.

REINERT J. & BAJAJ Y. P. S. (eds.) 1977 *Plant Cell, Tissue and Organ Culture*. Springer-Verlag, Berlin.

REY H.Y. & MROGINSKI L.A. 1978 *In vitro* culture of caulinary apices of manioc (*Manihot esculenta*). Phyton. 36, 171-176.

REYNOLDS B.D., BLACKMON W.J. & POSTEK C.E. 1980 Production of somatic embryos from Roselle callus. HortScience 15, 432.

RICE R.D., ALDERSON P.G. & WRIGHT N.A. 1983 Induction of bulbing of a tulip shoots *in vitro*. Sci. Hortic. 20, 377-390.

RIGHETTI B., MAGNANINI E. & MACCAFERRI M. 1988 Ethylene and other volatile substances produced by *in vitro*-cultured *Prunus avium*. Acta Hortic. 227, 402-404.

RIGHETTI B., MAGNANINI E., INFANTE R. & PREDIERI S. 1990 Ethylene, ethanol, acetaldehyde and carbon dioxide released by *Prunus avium* shoot cultures. Physiol. Plant. 78, 507-510.

RIJHWANI S.K. & SHANKS J.V. 1998 Effect of elicitor dosage and exposure time on biosynthesis of indole alkaloids by *Catharanthus roseus* hairy root cultures. Biotechnol. Prog. 14, 442-449.

RINGE F. 1972 Forderung der Zellproliferation unter der Einwirkung von 2-Chlorathylphosphorsaure bei Explantaten von *Begonia* x *richmondensis* in steriler Kultur. Z. Pflanzenphysiol. 67, 45-48.

RIOV J. & YANG S.F. 1982a Autoinhibition of ethylene production in citrus peel discs. Suppression of 1-aminocyclopropane-1-carboxylic acid synthesis. Plant Physiol. 70, 687-690.

RIOV J. & YANG S.F. 1982b Stimulation of ethylene production in *Citrus* leaf discs by mannitol. Plant Physiol. 70, 142-146.

ROBBINS J.A., KAYS S.J. & DIRR M.A. 1981 Ethylene and its role in the rooting of wounded mung bean cuttings. HortScience 16, 401.

ROBERTS L.W. 1969 The initiation of xylem differentiation. Bot. Rev. 35, 201-250.

ROBERTS L.W. & FOSKET D.E. 1966 Interaction of gibberellic acid and indoleacetic acid in the differentiation of wound vessel members. New Phytol. 65, 5-8.

ROBIE C.A. & MINOCHA S.C. 1989 Polyamines and somatic embryogenesis in carrot. I. The effects of difluoromethylornithine and difluoromethylarginine. Plant Sci. 65, 45-54.

ROBINSON K.E.P. & ADAMS D.O. 1987 The role of ethylene in the regeneration of *Helianthus annuus* (sunflower) plants from callus. Physiol. Plant. 71, 151-156.
RODRIGUEZ F.I., ESCH J.J., HALL A.E., BINDER B.M., SCHALLER, G.E. & BLEECKER A.B. 1999 A copper cofactor for the ethylene receptor ETR1 from *Arabidopsis*. Science 283, 996-998.
ROHRIG H., SCHMIDT J., WALDEN R., CZAJA I., MIKLASEVICS E., WIENEKE U., SCHELL J. & JOHN M. 1995 Growth of tobacco protoplasts stimulated by synthetic lipo-chitooligosaccharides. Science 269, 841-843.
ROSATI P., MARINO G. & SWIERCZEWSKI C. 1980 *In vitro* propagation of Japanese plum (*Prunus salicina* Lindl cv. Calita). J. Am. Soc. Hortic. Sci. 105, 126-129.
ROUSTAN J.P., LATCHÉ A. & FALLOT J. 1992 Enhancement of shoot regeneration from cotyledons of *Cucumis melo* by $AgNO_3$, an inhibitor of ethylene action. J. Plant Physiol. 140, 485-488.
RUBERY P.H. 1990 pp.119-146 in Roberts J., Kirk C.J. & Venis M.A. (eds.) *Hormone receptors and signal transduction in animals and plants.* Society for Experimental Biology. Symposium 44 The Company of Biologists Ltd. Cambridge UK .
RUBLUO A.M, KARTHA K.K., MROGINSKI L.A. & DYCK J. 1984 Plant regeneration from pea leaflets cultured *in vitro* and genetic stability of regenerants. J. Plant Physiol. 117, 119-130.
RÜCKER W. 1982 Combined influence of indolacetic acid, gibberellic acid and benzylaminopurine on callus and organ differebntiation in *Digitalis purpurea* leaf explants. Z. Pflanzenphysiol. 107, 141-151.
RUGINI E., BAZZOFFIA A. & JACOBONI A. 1988 A simple *in vitro* method to avoid the initial dark period and to increase rooting in fruit trees. Acta Hortic. 227, 438-440.
RYAN C.A. 2000 The systemin signaling pathway: differential activation of plant defensive genes. Biochim. Biophys. Acta - Protein Struct. Molec. Enzym. 1477, 112-121.
RYU S.B., LI P.H. & BRENNER M.L. 1988 Purification and estimation of ABA from potato cell suspension HortScience 23 754.
SAHOO Y., PATTNAIK S.K. & CHAND P.K. 1997 *In vitro* clonal propagation of an aromatic medicinal herb *Ocimum basilicum* L. (sweet basil) by axillary shoot proliferation. In Vitro Cell. Dev. Biol.-Plant 33, 293-296.
SANCHEZ-GRAS M.C. & SEGURA J. 1988 Morphogenesis *in vitro* of *Sideritis angustifolia*: effect of auxins, benzyladenine and spermidine. Plant Sci. 57, 151-158.
SANDERS I.O., SMITH A.R. & HALL M.A. 1991 Ethylene binding in epicotyls of *Pisum sativum* L. cv. Alaska. Planta 183, 209-217.
SANGWAN R.S., NORREEL B. & HARADA H. 1976 Effects of kinetin and gibberellin A3 on callus growth of orange formation in *Limnophila chinensis* tissue culture. Biol. Plant. 18, 126-131.
SANKHLA D., DAVIS T.D. & SANKHLA N. 1993 Effect of gibberellin biosynthesis inhibitors on shoot regeneration from hypocotyls explants of *Albizzia julibrissin*. Plant Cell Rep. 13, 115-118.
SANKHLA D., DAVIS T.D., SANKHLA N. & UPADHYAYA A. 1994 *In vitro* production of flowering shoots in German red carnation - Effect of uniconazole and gibberellic acid. Plant Cell Rep. 13, 514-518.
SANKHLA N. & SANKHLA D. 1968 Abscisin II-kinetin antagonism in growth of *Ipomaea* cotyledonary callus. Naturwiss. 55, 51-55.
SANTAMARIA J.M., MURPHY K.P., LEIFERT C. & LUMSDEN P.J. 2000 Ventilation of culture vessels. II. Increased water movement rather than reduced concentrations of ethylene and CO_2 is responsible for improved growth and development of *Delphinium in vitro*. J. Hortic. Sci. Biotech. 75, 320-327.
SAUERBREY P., GROSSMANN K. & JUNG J. 1987 Is ethylene involved in the regulation of growth of sunflower cell suspension cultures? J. Plant Physiol. 127, 471-479.
SAVAGE A.D., KING J. & GAMBORG O.L. 1979 Recovery of a pantothenate auxotroph from a cell suspension culture of *Datura innoxia* Mill. Plant Sci. Lett. 16, 367-376.
SCHALLER H., BOUVIER-NAVE P. & BENVENISTE P. 1998 Overexpression of an Arabidopsis cDNA encoding a sterol-C24(1)-methyltransferase in tobacco modifies the ratio of 24-methyl cholesterol to sitosterol and is associated with growth reduction. Plant Physiol. 118, 461-469.
SCHOTT H.-H. & SCHRAUDOLF H. 1967 Die wirkung einiger derivate der 9-fluorenol-9-carbonsaure auf die regeneration von begonienblattscheiben *Begonia rex*. Z. Pflanzenphysiol. 56, 387-396.
SCHRAUDOLF H. & REINERT J. 1959 Interaction of plant growth regulators in regeneration processes. Nature 184, 465-466.
SCHROEDER C.A. & SPECTOR C. 1957 Effect of gibberellic acid and indoleacetic acid on growth of excised fruit tissue. Science 126, 701-702.
SCORZA R., WELKER W.V. & DUNN L.J. 1982 Effect of glyphosate N-(phosphonomethyl)glycine on *in vitro* shoot regeneration and development in cranberry (*Vaccinium macrocarpon* Ait.). HortScience 17, 530.
SCORZA R., WELKER W.V. & DUNN L.J. 1984 The effects of glyphosate, auxin and cytokinin combination on *in vitro* development of cranberry node explants. HortScience 19, 66-68.
SEKIOKA T.T. & TANAKA J.S. 1981 Differentiation in callus cultures of cucumber (*Cucumis sativus* L.). HortScience 16, 451.
SHEPARD J.F. 1980 Abscisic acid-enhanced shoot initiation in protoplast-derived calli of potato. Plant Sci. Lett. 18, 327-333.
SHOEB F., YADAV J.S., BAJAJ S. & RAJAM M.V. 2001 Polyamines as biomarkers for plant regeneration capacity: improvement of regeneration by modulation of polyamine metabolism in different genotypes of *indica* rice. Plant Sci. 160, 1229-1235.
SHOEMAKER R.C., ATHERLY A.G. & PALMER R.G. 1983 Inhibition of deoxyribonuclease activity associated with soybean chloroplasts. Plant Cell Rep. 2, 98-100.
SCHOLL R.L., KEATHLEY D.E. & BARIBAULT T.J. 1981 Enhancement of root formation and fertility in shoots regenerated from anther- and seedling-derived callus cultures of *Arabidopsis thaliana*. Z. Phlanzenphysiol. 104, 225-231.
SHOYAMA Y., KAMURA K. & NISHIOKA L. 1988 Somatic embryogenesis and clonal multiplication of *Panax ginseng*. Planta Med. 54, 155-156.
SIMMONDS J.A. & WERRY T. 1987 Liquid-shake culture for improved micropropagation of *Begonia* x *hiemalis*. HortScience 22, 122-124.
SISLER E.C. & SEREK M. 1997 Inhibitors of ethylene responses in plants at the receptor level: recent developments. Physiol. Plant. 100, 577-582.
SISLER E.C. 1982 Ethylene binding properties of a Triton X-100 extract of mung bean sprouts. J. Plant Growth Regul. 1, 211-218.
SISLER E.C. 1991 Ethylene-binding components in plants. pp. 81-99 in Mattoo A.K. and Suttle J.C. (eds.) *The Plant Hormone Ethylene*, CRC Press, Boca Raton, Florida
SISLER E.C., DUPILLE E. & SEREK m. 1996 effect of 1-methylcyclopropene and methylcyclopropane on ethylene binding and ethylene action on cut carnations. Plant Growth Regul. 18, 79-86
SKOOG F. (ed.) 1980 *Plant Growth Substances*. Proc. 10^{th} Int. Conf. Plant Growth Substances. Madison, Wisconsin, 1979. Springer-Verlag, Berlin, Heidelberg..

SLOCUM R.D., KAUR-SAWHNEY R. & GALSTON A.W. 1984 The physiology and biochemistry of polyamines in plants. Archiv. Biochem. Biophys. 235, 283-303.

SMALLE J., HAEGMAN M., KUREPA J., VAN MONTAGU M. & VAN DER STRAETEN D. 1997 Ethylene can stimulate *Arabidopsis* hypocotyl elongation in the light. Proc. Natl. Acad. Sci.USA 94, 2756-2761.

SMITH D.L. & KRIKORIAN A.D. 1990 Somatic proembryo production from excised wounded zygotic carrot embryos on hormone-free medium; evaluation of the effects of pH, ethylene and activated charcoal. Plant Cell Rep. 9, 34-37.

SMITH D.R. & THORPE T.A. 1975 Root initiation in cuttings of *Pinus radiata* II. Growth regulation interactions. J. Exp. Bot. 26, 193-302.

SMITH M.A., DAVIES P.J. & REID J.B. 1985 Role of polyamines in gibberellin-induced internode growth in peas. Plant Physiol. 78, 92-99.

SMITH T.A. & WILTSHIRE G. 1975 Distribution of cadaverine and other amines in higher plants. Phytochemistry 14, 2341-2346.

SMITH T.A. 1977 Recent advances in the biochemistry of plant amines. Prog. Phytochem. 4, 27-81.

SMITH T.A. 1985 Polyamines. Annu. Rev. Plant Physiol. 36, 117-143.

SMITH T.A., ABBOTT A.J., BEST J.R. & CLEMENTS E. 1977 Polyamines in Paul's Scarlet rose cell suspension. p. 62 in Long Ashton Annu. Report 1976.

SNIR I. 1982 *In vitro* micropropagation of sweet cherry cultivars. pp. 735-736 in Fujiwara (ed.) 1982 (q.v.).

SOFFER H. & BURGER D.W. 1988 Studies on plant propagation using the aero-hydroponic method. Acta Hortic. 230, 261-269.

SONDAHL M.R., NAKAMURA T. & SHARP W.R. 1985 Propagation of coffee. pp 215-232 in Henke *et al.,* (eds.) 1985 (q.v.).

SONDHEIMER E., TZOU D.S. & GALSON E.C. 1968 Abscisic acid levels and seed dormancy. Plant Physiol. 43, 1443-1447.

SONGSTAD D.D., ARMSTRONG C.L. & PETERSEN W.L. 1991 $AgNO_3$ increases type II callus production from immature embryos of maize inbred B73 and its derivatives. Plant Cell Rep. 9, 699-702.

SPECTOR M. 1989 Method for protecting plants and plant matter from stress. US Patent No. 4802905.

SPIEGEL-ROY P. & KOCHBA J. 1980 Role of polyembryony and apomixis in *Citrus* propagation and breeding. pp 28-48 in Fiechter A. (ed.) 1980 *Advances in Biochemical Engineering*. 16 Soringer-Verlag, Berlin, Heidelberg, New York.

SPIEGEL-ROY P. & SAAD S. 1986 Effect of carbohydrates and inhibitors of GA_3 biosynthesis on embryogenic potential of salt tolerant and non-tolerant callus lines or orange (*Citrus sinensis* Osbeck). Plant Sci. 47, 215-220.

SRIVASTAVA S.K. & SMITH T.A. 1982 The effect of some oligo-amines and guanidines on membrane permeability in higher plants. Phytochemistry 21, 997-1008.

STASOLLA C., KONG L., YEUNG E.C. & THORPE T.A. 2002 Maturation of somatic embryos in conifers: Morphogenesis, physiology, biochemistry, and molecular biology. In Vitro Cell. Dev. Biol.-Plant 38, 93-105.

STEINITZ B. & YAHEL H. 1982 *In vitro* propagation of *Narcissus tazetta*. HortScience 17, 333-334.

STEPANOVA A.N. & ECKER J.R. 2000 Ethylene signaling: from mutants to molecules. Curr. Opin. Plant Biol. 3, 353-360.

STIMART D.P. 1986 Commercial microprop-agation of florist flower crops. pp. 301-315 in Zimmerman R.H., Griesbach R.J., Hammerschlag F.A. & Lawson R.H. (eds.) 1986 *Tissue Culture as a Plant Production System for Horticultural Crops*. Martinus Nijhoff, Dordrecht, Boston, Lancaster. ISBN 90-247-3378-2.

STUART R. & STREET H.E. 1971 Studies on the growth in culture of plant cells. J. Exp. Bot. 22, 96-106.

SUGE H.1972 Mesocotyl elongation in Japonica rice: effect of high temperatere pre-treatment and ethylene. Plant Cell Physiol. 13, 401-405.

SURESH M.R., RAMAKRISHNA S. & ADIGA P.R. 1978 Regulation of arginine decarboxylase and putrescine level in *Cucumis sativa* cotyledons Phytochem. 17, 57-63.

SWIATEK A., LENJOU M., VAN BOCKSTAELE D., INZÉ D., & VAN ONCKELEN H. 2002. Differential effect of jasmonic acid and abscisic acid on cell cycle progression in tobacco BY-2 cells. Plant Physiol. 128: 201-211.

SYONO K. & FURUYATA T. 1971 Effects of temperature on cytokinin requirement of tobacco calluses. Plant Cell Physiol. 12, 61-71.

TAEB A.G. & ALDERSON P.G. 1987 Micropropagation of tulip: optimizing shoot production from floral stem explants. Acta Hortic. 212, 677-681.

TAKAYAMA S. & MISAWA M. 1980 Differentiation in *Lilium* bulb scales *in vitro*. Effects of activated charcoal, physiological age of bulbs and sucrose concentration on different-iation and scale leaf formation *in vitro*. Physiol. Plant. 48 121-125.

TALMON L., VEGA M., MUJICA B. & BOLAND B. 1989 Cytohistological studies on the action of vitamin D3 and stigmasterol on *Phaseolus vulgaris* growing *in vitro*. Plant Sci. 59, 183-190.

TANG W. & FAN Q.Y. 1999 Plant regeneration via organogenesis from six families of loblolly pine. Plant Cell Tissue Organ Cult. 58, 223-226.

TANIMOTO S., MIYAZAKI A. & HARADA H. 1985 Regulation by abscisic acid of *in vitro* flower formation in *Torenia* stem segments. Plant Cell Physiol. 26, 675-682.

TAYLOR A.G. & WARHOLIC D.T. 1987 Protecting fluid drilled lettuce from herbicides by incorporating activated charcoal into gels. J. Hortic. Sci. 62, 31-37.

TAYLOR H.F. & BURDEN R.S. 1972 Xanthoxin, a recently discovered plant growth inhibitor. Proc. Roy. Soc. Lond. 180B, 317-346.

TAYLOR P.W.J., KO H.L., FRASER T.A., MASEL N. & ADKINS S.W. 1994 Effect of silver nitrate on sugarcane cell suspension growth, protoplast isolation, ethylene production and shoot regeneration from cell suspension cultures. J. Exp. Bot. 45, 1163-1168.

THEILER R. 1977 *In vitro* culture of shoot tips of *Pelargonium* species. Acta Hortic. 78, 403-409.

THOMAS D. des & MURASHIGE T. 1979a Volatile emissions of plant tissue cultures. I. Identification of the major components. In Vitro 15, 654-658.

THOMAS D. des & MURASHIGE T. 1979b Volatile emissions of plant tissue cultures. II. Effects of auxin 2,4-D on production of volatiles in callus cultures. In Vitro 15, 659-663.

THOMAS M.J. & TRANVAN H. 1982 Influence relative de la BAP et de l'IBA sur la néoformation de bourgeons et de racines sur les plantules du *Biota orientalis* (Cupressacées). Physiol. Plant. 81, 681-683.

THORPE T.A. & MEIER D.D. 1973 Effects of gibberellic acid and abscisic acid on shoot formation in tobacco callus cultures. Physiol. Plant. 29, 121-124.

THORPE T.A., HUXTER T.J. & REID D.M. 1981 The role of ethylene in shoot formation in tobacco callus. Plant Physiol. 67, (Suppl.) 27.

TIBURCIO A.F., FIGUERAS X., CLAPAROLS T., SANTOS M. & TORNÉ J.M. 1991 Improved plant regeneration in maize callus cultures after pretreatment with DL-α-difluoromethyl-arginine. Plant Cell Tissue Organ Cult. 27, 27-32.

TIBURCIO A.F., GENDY C.A. & TRAN THANH VAN K. 1989 Morphogenesis in tobacco subeepidermal cells: putrescine as amarker of root differentiation. Plant Cell Tissue Organ Cult. 19, 43-54.

TIBURCIO A.F., KAUR-SAWHNEY R. & GALSTON A.W. 1987 Polyamines and organogenesis in thin layer tobacco tissue cultures. pp. 43-56 in Jackson *et al.,* (eds.) 1987 (q.v.).

TISSERAT B. & MURASHIGE T. 1977a Effects of ethephon, ethylene and 2,4-dichlorophenoxyacetic acid on asexual embryogenesis *in vitro*. Plant Physiol. 60, 437-439.

TISSERAT B. & MURASHIGE T. 1977b Probable identity of substances in citrus that repress asexual embryogenesis. In Vitro 13, 785-789.

TISSERAT B. & MURASHIGE T. 1977c Repression of asexual embryogenesis *in vitro* by some plant growth regulators. In Vitro 13, 799-805.

TISSERAT B., ESAN E.B. & MURASHIGE T. 1979 Somatic embryogenesis in angiosperms. Hort. Rev. 1, 1-78.

TISSERAT B., ULRICH J.M. & FINKLE B.J. 1981 Cryogenic preservation and regeneration of date palm tissue. HortScience 16, 47-48.

TIZIO R., SAUSSAY R. & GAUTHERET R. 1970 Action de quelques gibberellins sur la rhizogenése de fragments de Topinambour cultivés *in vitro*. Compt. Rend. Acad. Sci. Paris 270D, 2088-2092.

TOKUJI, Y. and KURIYAMA, K. 2003 Involvement of gibberellin and cytokinin in the formation of embryogenic cell clumps in carrot (*Daucus carota*). J. Plant Physiol. 2, 133-141

TORRES K., TORRES J. & NATARELLA N.J. 1981 The production of gases by various plant species grown *in vitro*. HortScience 16, 441.

TORRIGIANI P., ALTAMURA M.M., CAPITANI F., FALASCA G., BAGNI N. & SARAFINI-FRACASSINI D. 1990 Inhibition of polyamine biosynthesis affects the rhizogenic response in tobacco thin layers. pp. 480-485 in Nijkamp *et al.,* (eds.) 1990 (q.v.).

TORRIZO L.B. & ZAPATA F.J. 1986 Anther culture in rice: IV. The effect of abscisic acid on plant regeneration. Plant Cell Rep. 5, 136-139.

TRAN THANH VAN K. & MUTAFTSCHIEV S. 1990 Signals influencing cell elongation, cell enlargement, cell division and morphogenesis. pp. 514-519 in Nijkamp *et al.,* (eds.) 1990 (q.v.).

TRAN THANH VAN K., TOUBART P., COUSSON A., DARVILL A.G., GOLLIN D.J., CHELF P. & ALBERSHEIM P. 1985 Manipulation of the morphogenic pathways of tobacco explants by oligosaccharins. Nature 314, 615-617.

TRAN THANH VAN M. 1973 Direct flower neoformation from superficial tissue of small explants of *Nicotiana tabacum* L. Planta 115, 87-92.

TWYFORD C.T. & MANTELL S.H. 1996 Production of somatic embryos and plantlets from root cells of the Greater Yam. Plant Cell Tissue Organ Cult. 46, 17-26.

TYAGI A.K., RASHID A. & MAHESHWARI S.C. 1980 Enhancement of pollen embryo formation in *Datura innoxia* by charcoal. Physiol. Plant. 49, 296-298.

VACHA G.A. & HARVEY R.B. 1927 The use of ethylene, propylene and similar compounds in breaking the rest period of tubers, bulbs, cuttings, and seeds. Plant Physiol. 2, 187-192.

VALLES M. & BOXUS Ph. 1987a Micropropagation of several *Rosa hybrida* L. cultivars. Acta Hortic. 212, 611-617.

VALLES M. & BOXUS Ph. 1987b Regeneration from *Rosa* callus. Acta Hortic. 212, 691-696.

VAN AARTRIJK J., BLOM-BARNHOORN G.J. & BRUINSMA J. 1985 Adventitious bud formation from bulb-scale explants of *Lilium speciosum* Thunb. *in vitro*. Effects of amino-ethoxyvinyl-glycine, 1-aminocyclopropane-1-carboxylic acid, and ethylene. J. Plant Physiol. 117, 401-410.

VAN GEYT J.P.C. & JACOBS M. 1985 Suspension cultures of sugarbeet (*Beta vulgaris* L.). Induction and habituation of dedifferentiated and self-regenerated cell lines. Plant Cell Rep. 4, 66-69.

VARGA M. & HUMPHRIES E.C. 1974 Root formation on petioles of detached primary leaves of dwarf bean (*Phaseolus vulgaris*) pretreated with gibberellic acid, triiodobenzic acid, and cytokinins. Ann. Bot. 38, 803-807.

VASIL V. & VASIL I.K. 1981 Somatic embryogenesis and plant regeneration from suspension cultures of pearl millet (*Pennisetum americanum*). Ann. Bot. 47, 669-678.

VENDRELL M. & McGLASSON W.B. 1971 Inhibition of ethylene production in banana fruit tissue by ethylene treatment. Aust. J. Biol. Sci. 24, 885-895

VENGADESAN G., GANAPATHI A., ANAND R.P. & ANBAZHAGAN V.R. 2000 *In vitro* organogenesis and plant formation in *Acacia sinuata*. Plant Cell Tissue Organ Cult. 61, 23-28.

VENGADESAN, G., GANAPATH, A., AMUTH, S. and SELVARAJ, N. 2003 High-frequency plant regeneration from cotyledon callus of *Acacia sinuata* (Lour.) Merr. In Vitro Cell. Dev. Biol.-Plant 39, 28-33

VENIS M.A. 1985 *Hormone Binding Sites in Plants*. New York, London: Longman.

VERNIERI P., PARDOSSI A. & TOGNONI F. 1991 Influence of chilling and drought on water relations and abscisic acid accumulation in bean. Aust. J. Plant Physiol. 77 25-28.

VINE S.J. & JONES O.P. 1969 The culture of shoot tips of hop (*Humulus lupulus*) to eliminate viruses. J. Hortic. Sci. 44, 281-284.

VINOCUR B., CARMI T., ALTMAN A. & ZIV M. 2000 Enhanced bud regeneration in aspen (*Populus tremula* L.) roots cultured in liquid media. Plant Cell Rep. 19, 1146-1154.

VON ARNOLD S. & ERIKSSON T. 1985 initial stages in the course of adventitious bud formation on embryos of *Picea abies*. Physiol. Plant. 60, 41-47.

VOYIATZI C. & VOYIATZIS D.G. 1988 shoot proliferation of the rose cv. (H.T.) Dr. Verhage as influenced by apical dominance regulating substances. Acta Hortic. 226, 671-674.

WALKER P.N., HEUSER C.W. & HEINEMANN P.H. 1988 Micropropagation: studies in gaseous environments. Acta Hortic. 230, 145-152.

WALLACE R.H. 1928 Histogenesis of intumescemces in the apple induced by ethylene gas. Am. J. Bot. 15, 509-524.

WALTERS D.R. 2000 Polyamines in plant-microbe interactions. Physiol. Mol. Plant Pathol. 57, 137-146.

WANG J.W., ZHANG Z. & TAN R.X. 2001 Stimulation of artemisinin production in *Artemisia annua* hairy roots by the elicitor from the endophytic *Colletotrichum* sp. Biotechnol. Lett. 23, 857-860.

WANG S.Y. & FAUST M. 1986 Effect of growth retardants on root formation and polyamine content in apple seedlings. J. Am. Soc. Hortic. Sci. 111, 912-917.

WAREING P.F. 1958 Interaction between indole-acetic acid and gibberellic acid in cambial activity. Nature 181, 1744-1745.

WEATHERHEAD M.A., BURDON J. & HENSHAW G.G. 1978 Some effects of activated charcoal as an additive to plant tissue culture media. Z. Pflanzenphysiol. 89, 141-147.

WEATHERHEAD M.A., BURDON J. & HENSHAW G.G. 1979 Effects of activated charcoal as an additive plant tissue culture media. Z. Pflanzenphysiol. 94, 399-406.

WEBB D.T., ARIAS W. & DE HOSTOS E. 1986 Callus formation by *Ginkgo biloba* embryos on hormone-free media controlled by closures and media components. Phytomorph. 36, 93-115.

WEINSTEIN L.H. & GALSTON A.W. 1988 Prevention of a plant disease by specific inhibition of fungal polyamine biosynthesis. US Patent No. 4818770.

WICKREMESINHE E.R.M., BLACKMON W.J. & REYNOLDS B.D. 1990 Adventitious shoot regeneration and plant produc-tion from explants of *Apios americana*. HortScience 25, 1436-1439.

WIDHOLM J.M., CHINNALA A.R., RYU J.H., SONG H.S., EGGETT T. & BROTHERTON J.E. 2001 Glyphosate selection of gene amplification in suspension cultures of three plant species. Physiol. Plant. 112, 540-545.

WILSON W.C. 1966 The possibilities of using ethylene gas to produce citrus fruit abscission under field conditions. Proc. Fla. State Hort. Soc. 79, 116-119.

WINATA L. & HARVEY R.G. 1980 Effect of glyphosate on organ formation in alfalfa callus *in vitro*. Plant Physiol. 65, (Suppl.) 89.

WOCHOK Z.S. & SLUIS C.J. 1980 Gibberellic acid promotes *Atriplex* shoot multiplication and elongation. Plant Sci. Lett. 17, 363-369.

WOCHOK Z.S. & WETHERELL D.F. 1971 Suppression of organized growth in cultured wild carrot tissue by 2-chloroethyl-phosphoric acid. Plant Cell Physiol. 12, 771-774.

WRIGHT N.A., ALDERSON P.G.& RICE R.D. 1982 Towards the clonal propagation of tulip by tissue culture. pp. 697-698 in Fujiwara (ed.) 1982 (q.v.).

WRIGHT S.T.C. & HIRON R.W.P. 1969 (+)-Abscisic acid, the growth inhibitor induced in detached wheat leaves by a period of wilting. Nature 224, 719-720.

WULSTER G. & SACALIS J. 1980 Effects of auxins and cytokinins on ethylene evolution and growth of rose callus tissue in sealed vessels. HortScience 15, 736-737.

YAHIA A., KEVERS C., GASPAR TH., CHENIEUX J.C., RIDEAU M. & CRECHE J. 1998 Cytokinins and ethylene stimulate indole alkaloid accumulation in cell suspension cultures of *Catharanthus roseus* by two distinct mechanisms. Plant Sci. 133, 9-15.

YANAGISAWA S., YOO S.-D. & SHEEN J. 2003 Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature 425, 521-525.

YANG S.F. & HOFFMAN N.E. 1984 Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35, 155-189.

YANG S.F., ADAMS D.O., LIZADA C., YU Y., BRADFORD K.G., CAMERON A.C. & HOFFMAN N.E. 1980 Mechanism and regulation of ethylene biosynthesis. pp. 219-229 in Skoog (ed.) 1980 (q.v.).

YANG Y.H., ZHANG H. & CAO R.Q. 1999 Effect of brassinolide on growth and shikonin formation in cultured *Onosma paniculatum* cells. J. Plant Growth Regul. 18, 89-92.

YATES P.J., LENTON J.R. & GOAD L.J. 1993 Effects of triazole inhibitors of sterol biosynthesis on the free and esterified sterol composition of celery cell-suspension cultures. Pesticide Sci. 39, 257-265.

YOKOTA T. 1999 Brassinosteroids. pp. 277-294 in Hooykaas *et al.,* (eds.) 1999 (q.v.).

YORK W.S., DARVILL A.G. & ALBERSHEIM P. 1984 Inhibition of 2,4-dichlorophenoxyacetic acid-stimulated elonga-tion of pea stem segments by a xyloglucan oligosaccharide. Plant Physiol. 75, 295-297.

YOUNG R.E., PRATT H.K. & BIALE J.B. 1951. Identification of ethylene as a volatile product of the fungus *Penicillium digitatum*. Plant Physiol. 26, 1074-1077.

YU Y.-B. & YANG S.F. 1979 Auxin-induced ethylene production and its inhibition by aminoethoxyvinylglycine and cobalt ion. Plant Physiol. 64, 1074-1077.

YUKIMUNE Y., TABATA H., HIGASHI Y. & HARA Y. 1996 Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in *Taxus* cell suspension cultures. Nature Biotechnol. 14, 1129-1132.

ZATYKO J.M., KISS J., RADICS Z. & SIMON I. 1989 Initiation of strawberry runner formation *in vitro*. Acta Hortic. 265, 349-352.

ZEEVART J.A.D. 1999 Abscisic acid metabolism and its regulation. pp. 189-207 in Hooykaas *et al.,* (eds.) 1999 (q.v.).

ZERONI M., GALIL J. & BEN-YEHOSHUA S. 1976 Autoinhibition of ethylene formation in nonripening stages of the fruit of sycamore fig (*Ficus sycomorus* L.). Plant Physiol. 57, 647-650.

ZHANG C.H. & WU J. Y. 2003 Ethylene inhibitors enhance elicitor-induced paclitaxel production in suspension cultures of *Taxus* spp. cells. Enz. Microb. Technol. 32, 71-77.

ZOBEL R.W. & ROBERTS L.W. 1978 effects of low concentrations of ethylene on cell division and cytodifferentiation in lettuce pith explants. Can. J. Bot. 56, 987-990

Chapter 8
Developmental Biology

1. INTRODUCTION

Unlike animals, higher plants continue to generate new organs and tissues throughout their post-embryonic life. This ongoing process is achieved through the activity of specific domains formed during embryogenesis at the two poles of developing embryos which have capacity to continuously renew themselves: the shoot and root apical meristems, respectively. The word "meristem" derives from the Greek "merizein", meaning "to divide" (Evans and Barton, 1997). Both shoot and root meristems add permanently new organs to the growing plant body and generate all the progeny cells that are required for tissue and organ differentiation. During the last decade, considerable progress has been made in the understanding of both shoot and root morphogenesis. A range of genes has been isolated from developmental mutants, namely from *Arabidopsis thaliana*, *Antirrhinum majus*, *Zea mays* and *Nicotiana tabacum*, and their temporal and spatial patterns of expression have been established using molecular biology techniques, so that we have now a better view of how shoot and root morphogenesis and development are controlled at cell, tissue and organ levels of organization. It is clear that each developmental step from embryogenesis to flowering, and each type of organ and tissue, is the result of the coordinated expression of sets of specific and developmentally-regulated genes. As a consequence for tissue culture, it can be expected a consideration of what genes are expressed in the initial explants, and how developmental genes are controlled by hormones and other components of culture media, would be helpful to improve the monitoring of *in vitro* manipulation. There is no doubt that the recent findings will have consequences for a better understanding of the processes of adventitious regeneration in tissue culture and the recently isolated genes will certainly serve as new tools to investigate these processes.

2. VEGETATIVE SHOOT MORPHOGENESIS

2.1. STRUCTURAL ORGANIZATION OF THE VEGETATIVE SHOOT APICAL MERISTEM

Although embryos from different species may vary in their developmental patterns and attain different sizes and degrees of differentiation (Esau, 1965), the future shoot apical meristem (SAM) becomes commonly recognizable at early stages during the establishment of the basic body of the embryo. It takes place in the apical domain of the globular embryo between the incipient cotyledonary primordia (Long and Barton, 1998; Lenhard and Laux, 1999) and the future SAM remains usually quiescent until germination (Pollock and Jensen, 1964). In the grass family and some dicotyledonous species, embryos become highly differentiated and the SAM becomes active and organized before germination. On the other hand, embryos from some parasitic herbs may be poorly differentiated and their SAM takes place entirely after germination (Esau, 1965). During the vegetative phase, i.e. the period during which leaves and stem are produced, the SAM varies in shape, size and meristematic activity. The SAM of conifers is commonly narrow and conical; it is narrow and elongated in some monocotyledons (wheat, *Elodea*) and dicotyledons (*Hippuris*) while it is dome-shaped, more or less broad and flat for other dicotyledons (*Arabidopsis*, tobacco). The shape and size of the shoot apex may also change during development (Nougarède, 1967; Besnard-Wibaut, 1975) and during a plastochron (Fig. 8.1), i.e. the time between the formation of successive leaves (Nougarède and Rembur, 1985). Whatever the species, some common features characterize the organization of functional SAMs of Angiosperms during the vegetative phase.

2.1.1. The layered organization of the SAM and its consequences for chimeras

The vegetative SAM is a highly ordered structure partitioned into domains with different functions. It usually displays an internal organization into cell layers (Fig. 8.2a) and zones (Fig. 8.2b). The layered appearance was early suggested by the analysis of chimeras (Satina *et al.,* 1940; Stewart and Dermen, 1975), the arrangement of cell layers being maintained by predominantly anticlinal cell divisions (plane of cell division perpendicular to the meristem's surface) in the outer cell layers. In most dicots, the SAM is composed of three lineages of cells termed histogenic layers: a two-layered tunica

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(L1, L2) surrounding a corpus (L3) in which one or more cell

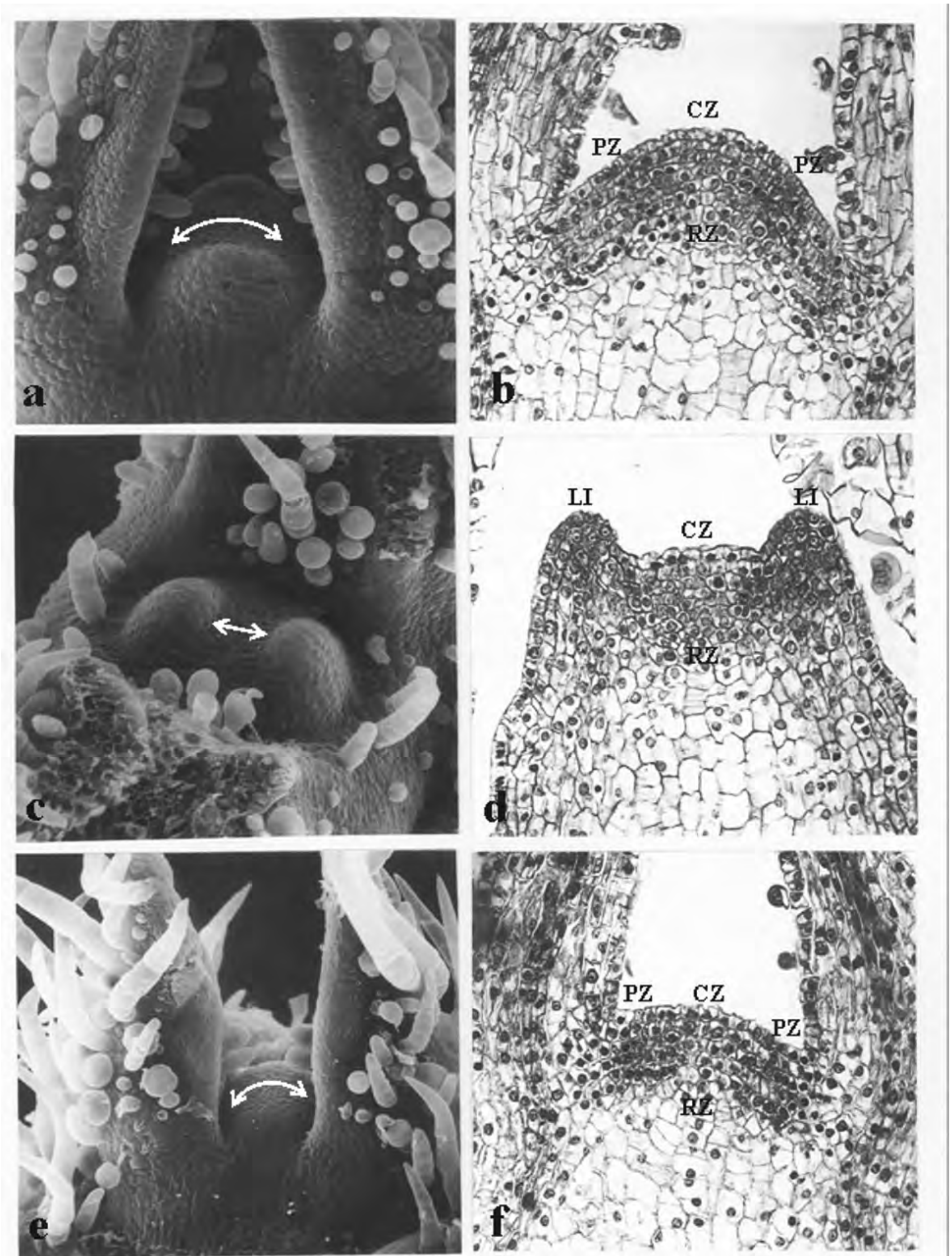

Fig. 8.1 – Morphological and structural evolution of *Coleus* vegetative shoot apical meristems during a plastochron.
a,b: maximal area before leaf initiation; c,d: minimal area just after leaf initiation e,f: regeneration phase preparing the future leaf initiation (courtesy of R. Saint-Côme 1985). a,c,e: scanning election microscopy; the arrows indicate the evolution of the SAM and the orientation of the corresponding longitudinal sections presented in b,d and f. CZ: central zone; LI: leaf initial; PZ: peripheral zone; RZ : rib zone.

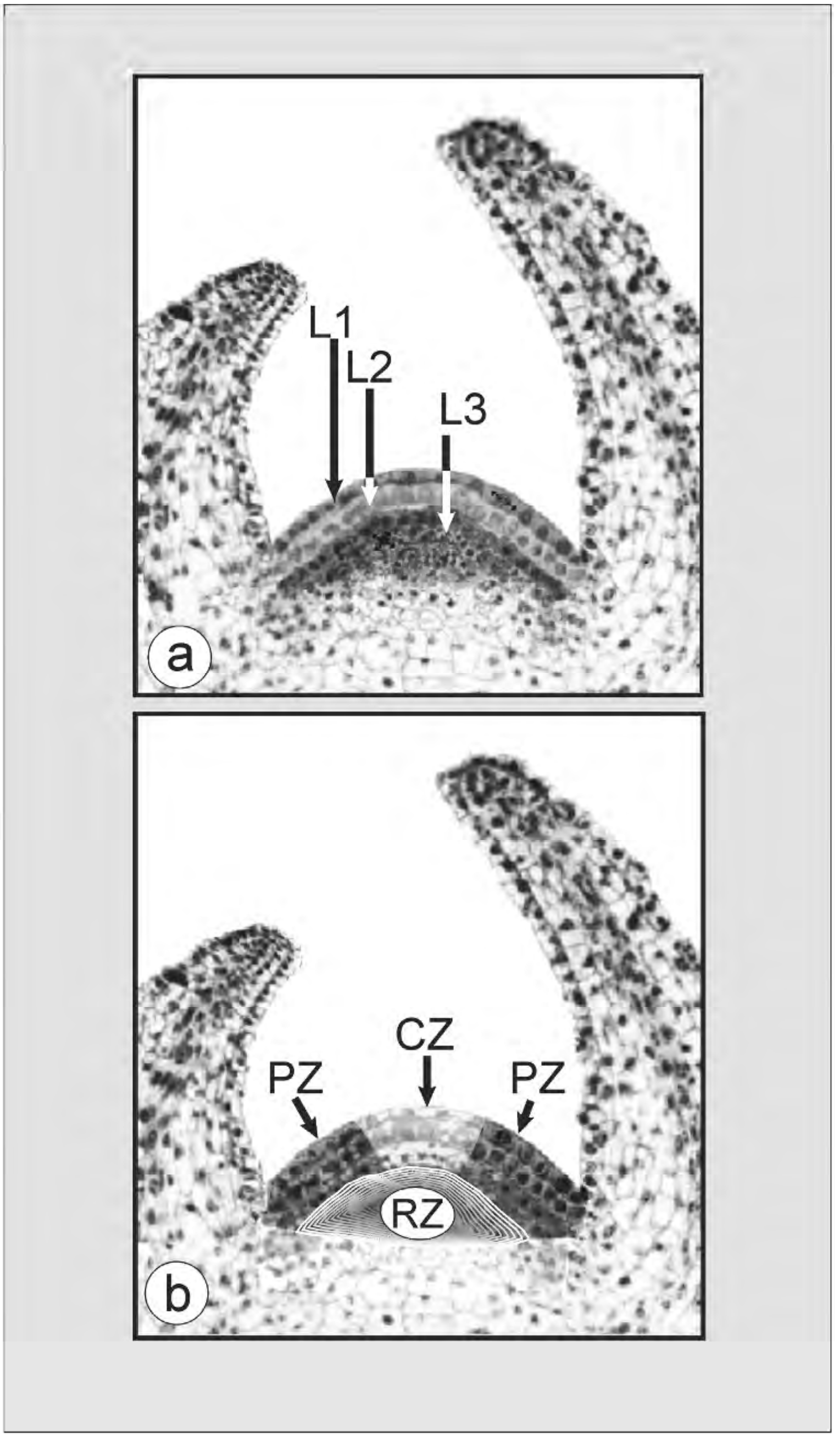

Fig. 8.2 – Layered (a) and zonated (b) structure of a tobacco vegetative shoot apical meristem. L1 –L3: layers; CZ: central zone; PZ: peripheral zone; RZ: rib zone (adapted from Bowman and Eshed, 2000, by N. Pépin, University P. M. Curie).

layers gives rise to the inner part, the pith rising from the lowest corpus layers. L1 generally divides only anticlinally and gives rise to the epidermis. The second layer, L2, forms part of the leaf mesophyll and is composed of cells dividing mainly in the anticlinal plane, except in zones where leaf initiation is occurring, and here the process involves periclinal division. The innermost corpus displays various division planes at the origin of the inner parts of leaves and stems, including the pith and the vascular strands. In monocots, both two- and three-layered apices are common. The control of orientation of cell division prevents the mixing of the layers during morphogenesis. Occasionally, abnormal division planes occur, forcing daughter cells into another layer (Laufs *et al.,* 1998c); this indicates that the developmental fate of plant cells is not strictly determined by cell lineage and that positional information and cell-to-cell communication are involved in patterning (Dawe and Freeling, 1991).

The particular layered organization can be disturbed during the process of adventitious bud formation, leading possibly to the loss of chimeric structures for horticultural species. Adventitious shoots can arise from tissues originating from L1 layer only, from L2 or L3 layers or from combinations of all three, and each layer does not necessarily keep its original fate during the process of axillary shoot formation (*in vivo*) or bud regeneration (*in vitro*). In addition, chimeras can originate from callus cultures composed of mixed populations of cells. Both these parameters have particular consequences for the vegetative propagation of plant chimeras, for example for maintaining leaf variegations through tissue culture. Three kinds of chimera are defined by the arrangement of the genetically different cells within the SAM. In *sectorial chimeras*, a wedge of genetically distinct cells is present through all apical cell layers. These chimeras arise most likely when mutations occur during the very early stages of embryo development. In *mericlinal chimeras*, part of one cell layer or more is genetically distinct from the remainder of the layer. These chimeras are unstable and tend to revert to periclinal chimeras or to become non-chimeral. In *periclinal chimeras*, one or more entire SAM layers is different from the other layers. Many horticultural plants are periclinal chimeras in which the genetic composition of SAM cell layers is different. These layers can be rearranged during cell proliferation and organization of the newly formed SAMs so that regenerated plants may contain a different chimeral composition or may no longer be chimeric. This leads to the idea that reversion of sports to normal and instability in periclinal chimeras could be due to "layer switching". In tobacco, it was demonstrated that the lineage of the three layers of the apical SAM was preserved in lateral meristems of first degree, but deviations of lineage were observed in secondary buds in which periclinal cell divisions destabilized the lineages (Tian and Marcotrigiano, 1993). Cells from one layer can divide periclinally and establish a new cell line in a different layer and this occurs in an uncontrolled manner (Fig. 8.3). Clearly the stability of chimeras depends upon how precisely the patterns of cell divisions in each meristem layer are maintained (Szymkoviak and Sussex, 1996) and this could probably be extended to the domain of micropropagation. In the limited advice that has been given on how to retain chimeral characteristics through tissue culture, it has been pointed out that chimeras can break down under conditions where the growth of one of the cell types is selectively favoured. Consequently, it was suggested that culture media composition may need to be modified to prevent any selective advantage. Progress in knowledge concerning the regulation of genes specifying each kind of cell layer, particularly the role of hormones on this regulation, will be certainly helpful for a better control of chimerical patterns in tissue culture. It must also be pointed out that positional information which is important at the whole plant level is probably highly perturbed in non-organised calluses submitted to shoot regeneration. Indirectly, the regeneration experiments with chimeras that lead to the perpetuation of chimeral patterns argue for the multicellular origin of adventitious shoots (Marcotrigiano and Gouin, 1984).

2.1.2. The cytophysiological zonation of the SAM

Superimposed on the layered organization, the active SAMs are provided with another pattern of organization called cytophysiological zonation (Fig. 8.2b). Cytochemical studies of SAM from various angiosperms revealed the presence of three zones (central or axial, peripheral or lateral and rib) provided with different staining patterns, planes of division and degrees of vacuolation (Nougarède,

1967; Gifford and Corson, 1971; Steeves and Sussex, 1989). Cells in the central zone stain less intensely with standard histological stains and are frequently more vacuolated. This central zone is not histologically distinguishable in some species. In other species, its detection depends on the stage of SAM development and the establishment of the SAM cytophysiological zonation is a secondary phenomenon in postembryonic development. The central zone has a slower rate of cell division than surrounding cells (Nougarède and Rembur, 1977, 1978).

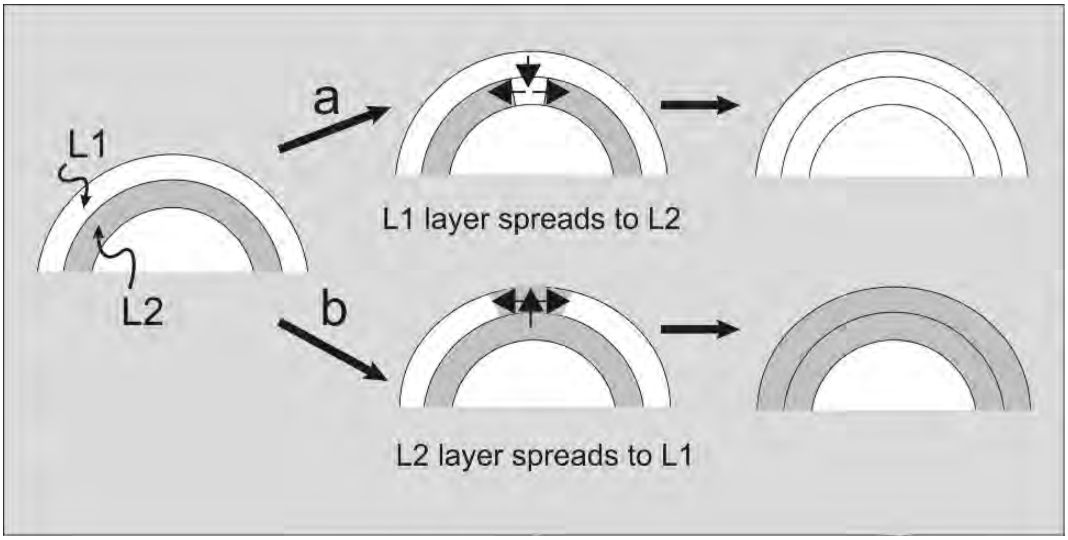

Fig. 8.3 – Possible explanation for chimeric instability through layer replacement (a: loss of an internal layer or displacement): (b: loss of the external layer). In both cases, the phenomenon starts from a typical cell division (periclinal instead of anticlinal).

The lateral zone ("initiating ring" according to Plantefol, 1947) surrounds the central zone and is the site of leaf formation involving a high rate of cell division. The rib zone is constituted of layers of flat cells subjacent to the central zone and gives rise to the central part of the stem. The rib zone is often absent in aquatic Angiosperms (Lance-Nougarède and Loiseau, 1960). It is well established that leaves develop on the flanks of the SAM in a defined arrangement whereas initial cells ("stem cells") reside in the central zone. The concept of stem cells is quite recent in plant biology. It is now accepted that a group of cells located in the basal part of the central zone of the SAM can reproduce themselves as well as generate differentiated progeny. Towards the end of a plastochron, just before the emergence of a distinct leaf primordium, the SAM is at its largest (maximal-area phase) while just after the initiation of a new leaf, the SAM is at its smallest (minimal area phase) (Fig. 8.4). The self-renewing population of initial cells resides within the central zone within which a small number divides slowly and produces new cells that are pushed out onto the flanks. These central cells are today considered as stem cells. According to the concept of the initiating ring, the lateral cells intensify their rate of cell division creating a leaf base (leaf founder cells) in positions related to the leaf arrangement (phyllotaxy). Successive sectors of the ring are visualized as being partly used up in the formation of leaves. Cell division restores each sector above a newly formed primordium so that the ring moves upward and the leaves arise at successively higher levels (Bersillon, 1955). During a plastochrone, the passage from the minimal area-phase to the maximal area-phase is allowed by anticlinal cell divisions that occur superficially (L1 and L2 layers) and enlarge the apical surface. Following this, periclinal divisions occur in the deeper layers, so that the lateral zone becomes thicker. The set of meristematic cells thus produced laterally forms the leaf base (leaf founder cells) from which leaf initiation occurs except for some rare cases in which leaves or leaf-like structures can develop in the absence of shoot meristem (Selker and Lyndon, 1996). Such cases can also be observed in tissue culture (Sattler and Maier, 1977). In most other instances, one of the earliest markers of leaf initiation from the peripheral zone is periclinal cell

division in the L2 layer, at the vicinity of procambial cells differentiated at an early stage within the leaf founder domain. Cells in the L1 and L3 adjust their growth accordingly, so the entire region acts in coordination to produce a leaf primordium (Bowman and Eshed, 2000). During periods of dormancy, the cytophysiological zonation is no longer recognizable and all the cells enter a quiescent state (Brossard, 1973; Cottignies, 1979).

The existence of at least two communicating compartments in the SAM, perhaps corresponding to the central and peripheral zones, was demonstrated by microinjection of fluorescent dyes into single epidermal cells of birch SAM and observation of the fluorescence spread (Rinne and van der Shoot, 1998). In addition, direct evidence that mRNA transcripts move from the body of the plant to the shoot apex through phloem long distance transport was reported, suggesting that higher plants could integrate developmental and physiological signals on a whole-plant basis (Ruiz-Medrano *et al.,* 1999).

2.2. MOLECULAR BASIS OF SAM IDENTITY AND ORGANIZATION

The view and the concepts concerning the functional organization of vegetative SAM and apical fate specification have been changed profoundly since the shoot apex was first recognized by Wolff (1759) and many series of genetic and molecular studies have been directed towards elucidating the complex process of SAM formation and functioning (Lehnard and Laux, 1999; Bowman and Eshed, 2000; Baurle and Laux, 2003; Laux, 2003; Veit, 2004). The resulting findings have provided molecular evidence supporting both the zoned and the stratified models. They are of fundamental interest for the understanding of the mechanisms of SAM behavior in plants but are also very helpful for the detection of early signs of SAM regeneration in tissue culture (Chee *et al.,* 2002; Cary *et al.,* 2002).

2.2.1. Genes involved in SAM establishment and maintenance

The main genes involved in SAM maintenance and organization encode either transcription factors or receptor/signal molecules (Table 8.1). Many of these genes belong to classes considered to be regulatory, controlling the expression of downstream genes. Thus current research is exploring the gene networks that underlie meristem function and cell fate determination. As in animals, families of plant homeobox genes have been found to play vital roles in developmental decisions that control cell specification and pattern formation. The complex gene expression patterns of the SAM, as shown by *in situ* approaches, develop gradually during embryogenesis. Early during embryo development, the expression of the shoot meristem-specific *SHOOT MERISTEMLESS* (*STM*) gene is observed between the incipient cotyledonary primordia and this gene is required for the production of a SAM (Long *et al.,* 1996).

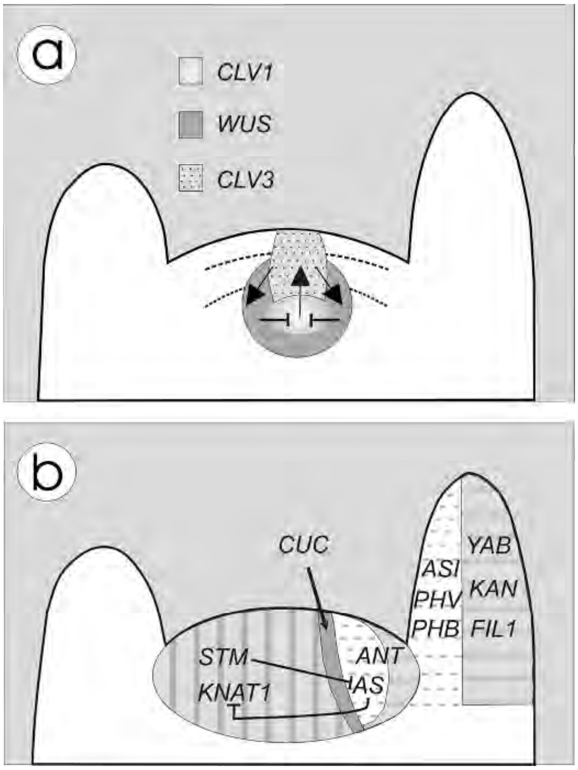

Fig. 8.4 – Genetic interactions in the shoot apical meristem. a: maintenance of the stem cells through the WUS/CLV auto-feed back loop; b: expression domains of selected genes involved in the establishment and patterning of leaf founder cells - for details of the genes noted in the figure, see Table 8.1 (adapted from Bowman and Eshed, 2000, by N. Pépin and E. Dubuisson, University P. M. Curie).

Table 8.1 – Genes playing a major role in initiation and maintenance of the shoot apical meristem (genes identified with *Arabidopsis thaliana* when the species is not otherwise indicated)

Genes	Gene products	Phenotypes of the loss-of-function mutants	Sites of expression in the SAM	Gene function	References
WUSCHEL (WUS))	WUS family of homeodomain transcription factor	Lack of central cells in the SAM and failure to sustain leaf organogenesis	Small subdomain of centrally located cells in the corpus (lower L3 cells) ; early expressed during embryogenesis (16-cell stage)	Maintenance of the population of undifferentiated central cells	Laux *et al.* (1996); Mayer *et al.* (1998); Schoof *et al.* (2000)
ZWILLE(*ZLL*)/*PINHEAD (PNH) ARGONAUTE1 (AGO1)*	AGO1-like proteins, found in many eucaryotes	Empty apices or presence of filamentous structures in place of shoots	Very early expressed during embryogenesis (4-cell stage) in provascular tissue then expression on the adaxial face of leaf primordia		Jürgens *et al.* (1994); Endrizzi *et al.* (1996); Moussian *et al.* (1998); Lynn *et al.* (1999)
KNOTTED1 (*KN1*, maize) *BREVIPEDICELLUS* (*BP*)/*KNAT1* *SHOOT MERISTEMLESS* (*STM*) *OSH1* (rice) *NTH15* (tobacco)	Class I KNOX family of homeodomain transcription factor	Lack of embryonic SAM or disorganized SAM with lack of maintenance	All the SAM cells excepted those participating to primordia formation in the peripheral zone *KN1* not expressed in layer L1 in *maize*	Maintenance of the SAM and establishment of the central-peripheral organization	Vollbrecht *et al.* (1991); Smith and Hake (1995); Barton and Poethig (1993); Jackson *et al.* (1994); Lincoln *et al.* (1994) ; Chuck *et al.* (1996); Clark *et al.* (1996); Endrizzi *et al.* (1996); Long *et al.* (1996); Sentoku *et al.* (1999) ; Ori *et al.* (2000)
CUP-SHAPED COTYLEDONS1 (*CUC1*)	Unknown	Fusion of cotyledons, sepals and stamen ; lack of embryonic SAM	Similar to *STM*	Shoot apical meristem formation;organ formation and separation; boundary maintenance; necessary for *STM* expression	Aida *et al.* (1997, 1999)
CUP-SHAPED COTYLEDONS2 (*CUC2*) *NO APICAL MERISTEM (NAM*, petunia*)*	NAC domain transcription factors (*CUC2 and NAM*)				

Table 8.1 – continued

Genes	Gene products	Phenotypes of the loss-of-function mutants	Sites of expression in the SAM	Gene function	References
CLAVATA1 (*CLV1*)	Transmembrane receptor kinase with a leucine-rich repeat (LRR) domain	Enlarged SAM through accumulation of cells in the central zone; increased number of leaves and flowers	Central zone in the L3 layer (site of the rib meristem), absent for L1 and L2	Regulation of the balance of undifferentiated central cells between proliferation and differentiation; control of the SAM size through restriction of proliferation in the *WUS* expressing region	Leyser and Furner (1992); Clark *et al.* (1993, 1996, 1997); Mayer *et al.* (1998); Fletcher *et al.* (1999); Lenhard and Laux (2003)
CLAVATA2 (*CLV2*)	LRR receptor kinase	Size increase of both vegetative and floral meristems, stem fasciation, extra-floral organs	nd		
CLAVATA3 (*CLV3*)	Secreted protein	As for *CLV2* + floral abnormalities	Central zone in the L1 and L2 tunica layers		
MGOUN1 and *2* (*MGO1* and *2*)	nd	Size increase of vegetative and floral meristems; decreased leaf number	nd	Control of the meristem size and of the number of lateral organs	Laufs *et al.* (1998a)

Plants homozygous for loss-of-function mutations in the *STM* gene have no SAM and produce seedlings with cotyledons, but no leaves are initiated, suggesting that cotyledons and leaves are not fully homologous organs as previously thought (Kaplan, 1969; Spurr, 1949). The *STM* gene is also required to prevent incorporation of central cells into lateral organ primordia (Endrizzi *et al.,* 1996). In *Petunia*, the gene *NO APICAL MERISTEM* (*NAM*) acts similarly to *STM* (Sauer *et al.,* 1996). The *STM* gene is a member of the *KNOTTED* class of homeodomain protein genes found in maize and soybean (Long *et al.,* 1996) and belongs to the KNOX family. Its expression marks the early initiation of SAM during embryogenesis and occurs in all types of SAM (vegetative, reproductive and axillary meristems) during development (Long *et al.,* 1996). It is required during vegetative growth to maintain the undifferentiated state of the central zone (Clark *et al.,* 1996; Endrizzi *et al.,* 1996). In maize, the *STM*-homologue gene *KNOTTED1* (*KN1*; Vollbrecht *et al.,* 1991) is also expressed in the SAM region of developing embryos (Smith *et al.,* 1995) but its expression is suppressed in the peripheral zone at sites involved in leaf initiation (Jackson *et al.,* 1994). The *KN1* gene appears to be a good marker of the undetermined state of the SAM cells. In maize, another homeobox gene (*RS1* for *ROUGH SHEATH 1*) has been identified and is also normally expressed in the SAM (Schneeberger *et al.,* 1995). Discovered by sequence homology and expression patterns, an *Arabidopsis* class 1 homeobox gene *KNAT1*, now known as *BREVIPEDICELLUS* (*BP*) (Lincoln *et al.,* 1994), could be an homolog of *RS1*. Some of the *KN1*-type homeobox gene family in rice (*OHS* genes) are also expressed in similar patterns (Sentoku *et al.,* 1999). As leaf initiation is correlated with a particular accumulation of endogenous cytokinins, as shown by *in situ* immunocytochemistry (Dewitte *et al.,* 1999; Guivarc'h *et al.,* 2002), it could be hypothesized that this hormone is involved in the cascade of regulation of the *KN1* gene family. *KNOX* genes also interact with gibberellins (Barley and Waites, 2002). In tobacco vegetative SAM, the *NTH20* gene, another related *knotted*-type homeobox gene, is expressed in the peripheral zone while the *NTH9* is expressed in the rib zone, and the *NTH1* and *NTH15* in the corpus (Nishimura *et al.,* 1999). *STM*

is also responsible for controlling expression of *UNUSUAL FLORAL ORGAN* (*UFO*) in a large band underlying the SAM (Long and Barton, 1998). *UFO* is involved in targeted protein degradation (Wang *et al.,* 2003) as well as in the induction of some floral genes.

Mutations of genes involved in SAM organization such as *WUSCHEL* (*WUS*) and *ZWILLE* (*ZLL*) affect the initiation of true leaves but not of cotyledons (Laux *et al.,* 1996; Moussian *et al.,* 1998). Mutation in the *WUS* gene leads to the formation of adventitious SAMs in the peripheral zone (Laux *et al.,* 1996), as does the destruction of the central zone by microsurgery experiments (Loiseau, 1959). This suggests that the central zone has an effect on the developmental potential of the peripheral zone and that the *WUS* gene is required to specify cells in the SAM central zone (Laux *et al.,* 1996). The *WUS* gene is expressed very early during embryo development before a SAM becomes evident. This expression is limited to a small group of L3 cells of the central zone (Fig. 8.4a). It has been proposed that *WUS*-expressing cells act as an organizing center, conferring stem-cell identity to the overlying initial cells (Mayer *et al.,* 1998). The *ZLL* gene is expressed when the provascular cells form at the 32-cell embryo stage and is specifically required to maintain the stem cells of the shoot meristem in an undifferentiated state and to mediate signaling from the vascular initial cells to the embryo apex (Moussian *et al.,* 1998). Interestingly, *ZLL* seems to act similarly to animal genes that regulate stem cell processes (Benfey, 1999b). In the *cup-shaped cotyledon* mutants (*cuc1, cuc2*), cotyledons are fused so that the *CUC* genes are considered to play a role in organ separation. During vegetative growth, the *CUC* genes are expressed in the boundary zone between the SAM and the leaf founder cells. However, they are also expressed all over the SAM during zygotic embryogenesis, indicating that they could also play a role in SAM establishment (Vroemen *et al.,* 2003). *CUC1* and *CUC2* genes encode NAC domain proteins that are highly homologous to the petunia NAM protein; *CUC1* may promote SAM formation by activating *STM* during embryogenesis (Takada *et al.,* 2001). In turn, *STM* influences *CUC1* and *CUC2* gene expression during embryo development. The involvement of *CUC* genes in controlling boundary size would be upstream - regulated by miRNAs (Laufs *et al.,* 2004).

Whether or not those genes involved in specifying the SAM identity are early expressed during *in vitro* SAM regeneration and at what time of the dedifferentiation process they begin to be involved has not yet been fully determined, nor how they could be regulated by hormonal factors of the media. However, it was shown that *KNAT1* or *KN1* overexpressing lines had increased potential for adventitious bud regeneration on leaves (Sinha *et al.,* 1993; Chuck *et al.,* 1996) and that *Arabidopsis* mutant lines, characterized by their capacity to form shooty callus in a hormone-autotrophic way, displayed increased mRNA levels of *KNAT1* and *STM*. This suggests that the shoot-forming phenotype is linked to altered cytokinin content or signaling (Frank *et al.,* 2000). Ectopic shoot production on leaves of transgenic tobacco and *Arabidopsis* lines over-expressing *KNOX* genes is not seen on monocot lines, suggesting that monocot leaves may have less developmental plasticity or are less competent to respond to the genes than leaves of dicots (Williams, 1998).

The stratified organization of the SAM has been confirmed by isolation of genes expressed specifically in distinct layers (Jenik and Irish, 2000). For instance, the homeobox gene *AtML1* is expressed in the L1 layer and clonally derived tissues, mainly the epidermis (Lu *et al.,* 1996; Sessions *et al.,* 1999). Other genes were also found to be highly expressed in the L1 layer and its derivatives. Examples are genes encoding lipid transfer proteins in carrot, tobacco and *Arabidopsis* (Sterk *et al.,* 1991; Fleming *et al.,* 1992; Thoma *et al.,* 1994), tomato polyphenol oxidase (Shahar *et al.,* 1992) or *Arabidopsis* protodermal factor 1 (Abe *et al.,* 1999). In contrast the *KNAT2* promoter is specifically expressed in the L3 layer of *Arabidopsis* SAM (Laufs *et al.,* 1998b and c). It was also shown that the KNOTTED protein is transported *via* plasmodesmata from the inner layers to the L1 while the corresponding mRNA can be detected only in the inner layers (Jackson *et al.,* 1994; Lucas *et al.,* 1995).

Interestingly, some genes identified as involved in regulating stem cell maintenance in plant meristems are homologous to those found in animals (Benfey, 1999b). The recent finding that human embryonic stem cells can be grown in culture will probably renew the interest in the plant field and the particular ability of plant cells to be grown in tissue culture. The above range of genes expressed in specific SAM layers or zones could provide helpful tools as markers of specific determined states to understand the process of direct and indirect bud regeneration *in vitro*. They would allow the study of the

characterization of competent cells in the initial explants or calluses and the timing of shoot determination, giving more precise information about the concepts of competence and expression. One might turn to further molecular work associated with careful stage-screened explants and *in situ* analyses for stage- and spatial-specific gene expression in relation to the inductive phytohormones.

2.2.1. Genes involved in SAM size and the question of fasciation

Another range of *Arabidopsis* genes has been reported to be involved in the control of SAM size. The loss-of-function *clavata1* mutation causes an increase in the size of both the shoot and floral SAM, leading to stem fasciation and the generation of flowers with extra floral organs (Leyser and Furner, 1992; Clark *et al.,* 1993). The *CLV1* gene encodes a leucine-rich repeat trans-membrane receptor serine/threonine kinase that suggests a role in cell signaling. *CLV1* is expressed in the central region of SAM corresponding to the rib zone; the *CLV1* mRNAs are not found in the L1 and L2 layers (Clark *et al.,* 1997). In the same gene family, *CLV3* is expressed throughout SAM development, predominantly in the L1 and L2 tunica layers of the region corresponding to the central zone (Fletcher and Meyerowitz, 2000). The stem cells are marked by the expression of *CLV3* (Fig. 8.4a); this signal has been hypothesised to inform the central zone cells either of their proper rate of cell division (Meyerowitz,1997) or of the rate at which they should exit the central zone to enter pathways leading to differentiation (Laufs *et al.,* 1998a). The *CLV* pathway functions as a negative feedback loop from the central stem cells to the organizing center (Fig. 8.4a; Schoof *et al.,* 2000). The signal is the small protein CLV3 which is secreted by the stem cells, moves intercellularly and activates a receptor that contains the CLV1 and CLV2 proteins to repress *WUS*. If the stem cells proliferate too much, increased CLV3 production diminishes the maintenance signal and keep the number of stem cells in check (Mar Castellano and Sablowski, 2005). In addition, the CLV1 receptor might limit the movement of the CLV3 protein and shield the *WUS* expressing domain from the negative effect of CLV3 (Lenhardt and Laux, 2003).

As the *Arabidopsis clavata3* mutant, the *mgoun1* and *2* mutants also display an enlarged vegetative SAM with occasional fasciation and accumulation of cells in the peripheral zone (Laufs *et al.,* 1998a); the corresponding *MGOUN* genes would affect the partitioning of peripheral cells into leaf or floral organ primordia. *MGOUN1* would encode a topo-isomerase involved in chromatin dynamics and stability. Indirect evidence indicates a probable hormonal control on the SAM fasciated phenotype but the effects of both auxin and cytokinins on the expression of meristem-specific genes remain to be elucidated. Old research (White, 1948; Gorter, 1965) described three types of abnormal shoots considered as fasciated: linear, circular and radiate. Fasciations are the result of the fusion of two or several growth points. In linear fasciations, the stem is flattened and the SAM is enlarged and flat as a ribbon (Ecole, 1970); such enlarged SAMs can be also observed in tissue culture in the presence of high cytokinin concentrations (Brossard, 1976). In such treatments, calluses surrounded with continuous meristematic bands not organized into functional SAMs or enlarged SAMs, can be observed dividing dichotomously into two normal SAMs (personal observation). In circular fasciations, SAMs are ring-shaped and such appearance can be observed following treatments with inhibitors of auxin transport (Ecole, 1971) or in the *pin1* mutant which is altered in auxin efflux (Vernoux *et al.,* 2000a). Inhibition of auxin transport can also result in fused leaf organs (Ecole, 1972) similar to what occurs in the *cuc* mutants, which are altered in organ separation. In the radiate fasciations, the SAM and the stem have a stellate shape in transverse section.

2.3. PHYLLOTAXY AND PATTERNS OF LEAF DEVELOPMENT

2.3.1. Phyllotaxy

Investigation of what determines the sites at which a new leaf is initiated in the SAM peripheral zone is in progress. Phyllotaxy, i.e. the positioning of leaves on the stem, has been hypothesised as a self-organized growth process (Douady and Couder, 1992) as leaf primordia normally arise in regular geometric arrays. However, these arrays result probably from the combination of genetic, environmental and developmental factors (Schwabe, 1984; Marc and Hackett, 1991). There are two classes of possible leaf arrangement: whorls (with opposite or decussate leaf position) and spirals. Several authors considered that the signal for leaf initiation could spread acropetally from the underlying tissues towards the point where a leaf is going to rise (inhibitory field theory) and that the extension of procambium differentiation may play a role in the phyllotaxic organization of the stem in determining the initiating sites of the leaves (Buvat,

1989). Phyllotaxy involves communication between existing and newly formed leaf primordia. The existence of signals from leaves to the SAM through symplastic continuity, and of specialized symplastic domains in the SAM itself, was observed by monitoring fluorescent symplastic tracers (Gisel *et al.,* 1999).

The signalling events involved in determination of when and where new leaf primordia are formed in the peripheral zone of the SAM are beginning to be elucidated. Studies using local application of the wall-loosening protein expansin to meristems have indicated the potential role of biophysical forces in the process of leaf initiation (Fleming *et al.,* 1997; Pien *et al.,* 2001). Leaf primordium formation by the SAM can also be induced by a local application of auxin (Reinhardt *et al.,* 2000). In addition, *LeEXP18*, a tomato gene encoding expansin, is auxin-inducible and expressed preferentially at the sites of future leaf initiation, particularly in the L1 cells, making this gene an early marker for the sites of leaf initiation (Reinhart *et al.,* 1998). The tobacco *NFL* gene, (a homolog of the snapdragon *FLORICAULA* (*FLO*) and *Arabidopsis LEAFY* (*LFY*) genes,) and the *Arabidopsis AINTEGUMENTA1* (*ANT1*) gene, are both expressed in young leaf primordia and in a SAM region that may correspond to the peripheral zone (Evans and Barton, 1997). In contrast, it has been shown that *STM*, *KNAT1*, *OSH1*, *OSH43* and *CLV1* genes are down-regulated in these sites (Table 8.1), suggesting that their control mechanism is involved in phyllotaxy, but further studies are necessary to clarify the phenomenon. It is noteworthy that many of the mutations that affect SAM size also affect phyllotaxy: *clv1*, *clv3* and *amp1* mutants have enlarged SAMs and all make leaves with abnormal spacing pattern, the latter being characterized by high cytokinin levels (Chaudhury *et al.,* 1993). Results from the *abphyl 1* maize mutant (Jackson and Hake, 1999) that displays both altered phyllotaxy and enlarged SAM, suggest a relation between meristem size and phyllotaxy. Lastly, some mutants modified in their plastochrone duration have also been isolated. For example, in the rice mutants *plastochrone-1* (*pla-1*, *pla-2*), plastochrone is shortened and an ectopic expression of the vegetative program is observed during the reproductive phase (Itoh *et al.,* 1998). In addition, studies of the *Arabidopsis pin1* mutant that possesses few leaves then a naked stem have confirmed the role of auxin in phyllotaxy. [PIN1 is a transmembrane protein with homologies to a family of auxin transporters and local auxin application restores leaf initiation from the naked stem (Reinhardt *et al.,* 2000 ; Vernoux *et al.,* 2000a)]. How polar transport of auxin can control phyllotaxy has been recently demonstrated: auxin is transported upwards into the meristem through the epidermis and the outermost L1 layer; existing leaves act as sinks, redistributing auxin and creating its heterogeneous distribution in the SAM; auxin accumulation occurs only at a certain distance from existing primordia, defining the position of future primordia (Reinhardt *et al.,* 2003; Fleming, 2004). A control of phyllotaxy could be exerted upstream of the auxin transport mechanism by the cytokinin-inducible response regulator homolog *ABPH1*, indicating a role for cytokinins in setting the field size within which the auxin-patterning system can function (Giulini *et al.,* 2004).

2.3.2. Genes involved in leaf organ identity

Leaf initiation involves an increased rate of proliferation of cells that are recruited from the SAM peripheral zone. All the SAM specific genes from the *KNOX* family have been found to be downregulated at sites where leaf initiation is in preparation (Fig. 8.4b), so they can be considered as negative markers of leaf determination. A range of genes that are positively regulated at the sites of leaf initiation have been also identified (Table 8.2). Appropriate expression of the *CUC* genes is necessary in the presumptive boundary cells to prevent the formation of fused organs but the mechanism by which these boundaries are set in relation to the predicted auxin maxima that define sites of leaf initiation is unknown. Early in leaf initiation, *ANT 1* (Elliot *et al.,* 1996; Barton, 1998) and *FIL* (Sawa *et al.,* 1999 ; Siegfried *et al.,* 1999) genes start to be expressed in the leaf founder cells and appear as positive markers. Another *Arabidopsis* transcription factor gene, *AS1*, is expressed early during leaf initiation (Byrne *et al.,* 2000) as are its homologs *PHAN* in *Antirrhinum* (Green, 1999) and *RS2* in maize (Tsiantis *et al.,* 1999). *STM* repressed the expression of *AS1*/*PHAN* which in turn represses the expression of *KNAT1* in the developing leaf primordia (Ori *et al.,* 2000). Close interactions between the SAM and its flanking regions seem to control the acquisition of leaf determination by the lateral cells (Fig. 8.4). This process is probably highly disturbed in some cases observed *in vitro* where leaf initiation seems to occur independently of the presence of organized SAMs.

Table 8.2 – Genes involved in leaf initiation and development.
(genes identified in *Arabidopsis thaliana* when no indication of the species is given)

Genes	Gene products	Mutant phenotypes	Sites of expression	Gene function	References
ASYMMETRIC LEAVES1 (*AS1*) *ROUGH SHEATH* (*RS2*, maize) *PHANTASTICA* (*PHAN*, *Antirrhinum*)	ARP type of MYB-domain transcription factors	Ectopic expression of *KNOTTED*-like genes in the leaf primordia; altered leaf development	Sites of leaf initiation (leaf founder cells) in the SAM peripheral zone (in places where *KN1* / *STM* are not expressed); developing vascular cells in the stem	Inactivation of *KNOTTED*-like genes; promotion of adaxial identity	Jackson *et al.* (1994); Waites *et al.* (1998); Tsiantis *et al.* (1999); Byrne *et al.* (2000)
ASYMMETRIC LEAVES2 (*AS2*)	Cysteine-repeat family of leucine zipper domain transcription factors	Id *AS1*		Normal development of lamina; limitation of *KNOX* gene expression	Wakawa *et al.* (2002)
AINTEGUMENTA (*ANT*)	Putative transduction factor (*APETALA 2* class DNA binding)	Altered flower development, slight reduction of leaf size	Cotyledon primordia during embryogenesis; leaf and flower organ primordia	Rapid cell division and growth of peripheral SAM cells giving rise to leaf primordia	Long and Barton (1998)
LEAFY (*LFY*) = *FLORICAULA* (*FLO*, *Antirrhinum*)	Transcription factor	Prolonged vegetative phase	Expressed early in leaf and floral primordia	Mainly involved in identity of floral organs	Mandel *et al.* (1992); Weigel *et al.* (1992); Vernoux *et al.* (2000a)
UNUSUAL FLOWER ORGAN (*UFO*) *FIMBRIATA* (*FIM*, *Antirrhinum*)	F-box proteins	Fused floral organs	Frontiers between floral organs, between floral meristems and according a SAM subregion	Organ separation	Ingram *et al.* (1995); Lee *et al.* (1997)
PIN-FORMED1 (PIN1) *PINOID (PID)*	Transmembrane protein (PIN1) with role in auxin transport Serine-threonine kinase (*PID*)	Reduced number of cotyledons; fused organs; inflorescence axis without flower primordia	*PIN*: lateral organs *PID*: frontiers between organs	Initiation and separation of organs *via* *CUC2* inactivation in the primordia	Galweiler *et al.* (1998); Vernoux *et al.* (2000a)
FILAMENTOUS FLOWER *(FLL)* *YABBY3* (*YAB3*)	Zinc finger domain transcription factors with a HMG box-like domain (*YABBY* gene family)		SAM peripheral zone under young leaf primordia; sites of floral primordia initiation; peripheral zone; abaxial side of leaf primordia	Maintenance and growth of floral and inflorscence meristem; promotion of abaxial cell fate in lateral organs; inactivation of KNOX genes	Sawa *et al.* (1999); Siegfried *et al.* (1999); Kumaran *et al.* (2002)

Table 8.2- continued

Genes	Gene products	Mutant phenotypes	Sites of expression	Gene function	References
KANADI1 (KAN1)		*Gain-of-function mutations*: abaxialization of the lateral organs and loss of meristem development *Loss-of-function mutations* : adaxialization of lateral organs; alteration of vascular patterning in the stems; expansion of *PHB*, *PHV* and *REV* expression domain	Developing phloem (abaxially), only in the root	Vascular patterning, antagonist to meristem function through interactions with HD-ZIP III genes	Eshed *et al.* (2001); Kerstetter *et al.* (2001)
KANADI2 (KAN2) *KANADI3 (KAN3)*			Developing phloem (abaxially), throughout the plant		
PHABULOSA (*PHB*) *PHAVOLUTA* (*PHV*)	HD-ZIP III family of homeodomain transcription factors; may be regulated by microRNAs	*Gain-of-function mutations*: Adaxialization of lateral organs; radialization of vascular bundles, ectopic SAMS on the abaxial side of leaves, loss of *YABBY* gene activity *Loss-of-function mutations*: Abaxialized cotyledons and no primary apical meristem	Throughout the embryonic SAM, in adaxial region of the cotyledon primordia and in vascular precursors of hypocotyls and root, in all apical meristems, in developing vasculature, in adaxial domain of lateral organs.	Regulation of apical embryo patterning and of lateral meristem initiation, regulation of leaf polarity	McConnell and Barton (1998)
REVOLUTA (*REV*)/ *INTERFASCICULAR FIBERLESS (IFL)/AMPHIVASAL BUNDLES(AVB)*		Aberrant axillary and flower meristem formation; defects in leaf development, stem cell specification, vascular development and auxin transport		Growth of apical meristems, limitation of leaves, floral organs and stems growth, fibre differentiation	Talbert *et al.* (1995); Otsuga *et al.* (2001)
ATHB8 *CORONA (CNA) /ATHB15*		*Loss-of-function mutations*: No aberrant phenotype	Vascular tissues (developing xylem, positioned adaxially)	Vascular development	Baima *et al.* (1995)

2.3.3. Genes controlling leaf dorsiventrality and final shape

Leaves are determined organs that grow to specified size and specific shape. One common feature of most leaves is that they display a bilateral symmetry and a dorsiventrality, i.e. an abaxial-adaxial asymmetry (abaxial being away from the SAM and adaxial being adjacent). This suggests that common genetic programs are involved to establish these features (Table 8.2). Surgical incisions separating incipient leaf primordia from the SAM suggested that the SAM may be the source of a signal required for proper polar development of the leaf (Sussex, 1955). The genetic basis of regulatory relationships between the SAM and lateral organs has been revealed by the analysis the *Antirrhinum* mutant *phantastica* (*phan*), which displays needle-shaped abaxialized leaves. The *PHAN* gene encodes a MYB-related transcription factor expressed throughout

lateral leaf primordia (Waites *et al.,* 1998). When grown under non-permissive conditions, the mutant plants develop radialized leaves with predominantly abaxial cell types and their SAM is arrested. The *hb* (*handlebar*) mutation enhances the effects of the *phan* mutation on dorsal fate in leaves, suggesting that both are additional (Waites and Hudson, 2001). In contrast, the *Arabidopsis* mutant *phabulosa-1d* displays enlarged SAM and produces leaves with adaxial cell types and ectopic buds (McConnell and Barton, 1998). The emerging picture is that factors both intrinsic and extrinsic to the leaf primordium contribute to the specification of cells as abaxial or adaxial (Fig. 8.4b). Genes form the *YABBY* family (genes encoding a helix-loop-helix/HMG-like region) including *FIL* and *YAB 3* genes were shown to be expressed specifically in a polar manner in all the organs (vegetative or floral) produced by the SAM and to be responsible for the specification of abaxial cell fate (Siegfried *et al.,* 1999). The *KANADI 1* (*KAN1*) gene is another of the key determinants of abaxial identity. *KAN 1* encodes a putative transcription factor but little is known about its involvement in abaxiality. *PHAN* is needed to specify adaxial identity and to repress adaxial expression of *YABBY* (Golz and Hudson, 2002). Evidence is also reported that the *PINHEAD* gene is also involved, defining a domain comprising the adaxial face of young leaves, and that this domain is essential for SAM development (Lynn *et al.,* 1999).

A correlation between the acquisition of adaxial leaf fate and the development of ectopic buds has been suggested (Mc Connell and Barton, 1998), that is in contrast with the common model in which axillary meristems are derived from remnant meristematic cells (so-called detached meristems) of the primary SAM. This possible link could have some relevance in tissue culture when bud regeneration is expected from a leaf explant. In the same way, somatic activation of an *ipt* transgene encoding cytokinin overproduction in tobacco led to transgenic lines provided with ectopic buds on the adaxial surface of their vascular strands (Estruch *et al.,* 1991) and the leaf explants of transgenic tobacco overexpressing the *KNAT1* gene give rise to a prolific bud regeneration along the veins of their adaxial surface (Pépin *et al.,* in preparation).

Different rates of expansion and cell division influence final leaf shape that can be simple (*Arabidopsis*, tobacco) or complex (tomato). Misexpression of *KNOX* genes in tomato results in disruption of compound leaf development, with phenotypes ranging from multiply ramified to filamentous (Hareven *et al.,* 1996; Kessler and Sinha, 2004). In tomato, but not in species with simple leaves, *KNOX* genes are expressed in the leaf primordia and play a role in compound leaf development. Surprisingly, a tomato homolog of *PHAN* is expressed in the SAM. In species that have simple leaves, expression of *PHAN* family members is excluded from the SAM, suggesting that interactions between *KNOX* genes and their regulators are different in species with compound leaves and in those with simple leaves. Formation of maize leaf margins is disrupted by the polar auxin transport inhibitor NPA together with the KNOX protein regulation (Scanlon, 2003). Another mechanism affecting compound leaf development involves the *Arabidopsis* floral meristem identity *LEAFY* (*LFY*) and its homologs *UNIFOLIATA* (*UNI*) in pea and *FLORICAULA* (*FLO*) in *Antirrhinum*. For example, in *uni* mutants, compound leaves are converted to simple (Höfer *et al.,* 1997).

Except in the rare cases in which leaves can arise independently of meristems (Schichnes *et al.,* 1997), interactions between the SAM and the developing leaf are required. The best example is the maintainence of silencing of *KNOX* genes in developing leaves by *PHAN* and its homologs. These genes may encode transcription factors required for maintaining epigenetic states; they also affect stem cell maintenance. In addition some of the genes involved in axis specification encode epigenetic regulators of gene expression such as RNAi or chromatin remodeling factors (Byrne *et al.,* 2001). In this way, recent advances have indicated that miRNAs act in the abaxial domain of the leaf primordia by silencing adaxializing transcription factors (Kidner and Martienssen, 2004).

2.4. DEVELOPMENT OF AXILLARY AND ADVENTITIOUS MERISTEMS

2.4.1. Axillary buds and lateral branching

During post-embryonic development, reiterative SAM functioning produces repeated units, often referred to as phytomers. The components of each unit are a leaf with one or more axillary buds and a stem portion comprising a node and an internode. The axillary buds are secondary shoot meristems located at the junction between the stem and the leaf base and responsible for lateral branching. This axillary branching generally involves two developmental stages: the formation of axillary meristems and the subsequent growth of the axillary bud. Axillary meristems can originate either at the

same time as the leaf primordia from a subset of cells of the SAM that retain meristematic properties (Garrison, 1955) or later from already differentiated tissues that regain meristematic traits (Esau, 1965; McConnell and Barton, 1998). In any case, they develop from superficial cells and are therefore exogenous in origin; only a few species are able to differentiate axillary buds directly from the epidermis. The initiation of the lateral buds is usually characterized by a combination of anticlinal divisions in one or more of the superficial layers and by various types of divisions in the deeper layers (Esau, 1965). *In situ* hybridization indicates that expression of *STM* gene is associated with the formation of *Arabidopsis* axillary meristems during vegetative development (Grbic and Bleecker, 2000). Sometimes, a series of curved layers is established at the base of the early bud meristem, forming a "shell-zone". This particular zone is characterized by high peroxidase and polyphenol oxidase activities (Brossard, 1975) but its physiological significance is not yet fully understood (Shah and Patel, 1972). This zone has been interpreted in terms of metabolic isolation of the developing axillary bud from the main SAM but also as an initial response of the axillary cells to the stresses that are set up in the axil due to the growth of the apex (Lintilhac and Vesecky, 1980).

Once initiated, the axillary shoot meristems may either develop into branches, or they may develop into an axillary bud in which growth arrests after a few leaf primordia have formed. The control of subsequent axillary bud growth is by the main shoot apex, the apical dominance of which becomes less as it grows on upward and becomes more distant from the bud. Apical dominance is also reduced when the plant enters the flowering process. When inhibited, pea axillary buds display a cell cycle arrest in a G0 presynthetic phase of the cell cycle (Nougarède *et al.,* 1981) and lack mRNA corresponding to the ribosomal protein L27 which is highly linked to the growing state (Stafström, 1995). They are metabolically active as growing buds but two-dimensional gel electrophoresis has shown that certain proteins are specifically expressed in either growing or dormant buds (Stafström, 1995).

In recent years, work with transgenic plants has shown that the endogenous alteration of the auxin/cytokinin balance as a consequence of the introduction of single dominant transgenes, profoundly influences lateral branching (Klee and Estelle, 1991; Medford *et al.,* 1989; Smigocki and Owens, 1989). This lends support to the so-called "hormone-balance theory" of apical dominance established by previous experimental treatments with exogenous phytohormones (Cline, 1994). Auxin and cytokinin appear to play a major role in controlling this process, with cytokinin as a key factor in promoting bud growth whereas auxin may have an inhibitory effect. In the succulent plant *Grapto-petalum*, the lower residual meristem at the base of the leaf does not produce buds but buds can be induced there by exogenous cytokinin, the size of the bud being determined by the size of the treated area. This suggests that the responding cells are only those receiving the stimulus directly (Lyndon, 1990). Transgenic tobacco plants expressing locally the *IPT* gene encoding cytokinin biosynthesis at the level of axillary buds, display a reduced apical dominance (Hewelt *et al.,* 1994; Guivarc'h *et al.,* 2002). Also early work on tomato mutants with reduced branching, such as *lateral suppressor* (*ls*) and *torosa,* has correlated this trait with a reduced cytokinin content, the *ls* mutant being devoid of any axillary meristems (Malayer and Guard, 1964; Sossountzov *et al.,* 1988). However, increased cytokinin production mediated by *IPT* gene transfer, failed to induce axillary bud formation on the *ls* mutant (Groot *et al.,* 1995). It has been shown that *ls* phenotype is caused by a loss of function of the VHIID family of regulatory proteins. Schümacher *et al.* (1999) speculated that LS protein may regulate the gibberellin signal transduction pathway as do other known protein members of this family. In addition, periclinal chimeras consisting of a genetically recessive *ls* L1 layer and wild-type L2 and L3 layers produced axillary meristems (Szymkowiak and Sussex, 1993), suggesting that a functional *LS* gene is required only in specific cell layers of the meristem and confirming the existence of signaling between the cell layers (Napoli *et al.,* 1999).

Only a few other mutants with altered axillary meristem development have been characterized. In maize, *TEOSINTE BRANCHED 1* repressed the growth of axillary buds (Deebley *et al.,* 1997). The pea *ramosus* mutants (*rms 2* and *rms 3*) display an increased branching and the mutation affects the levels of graft-transmissible substances that inhibit branching, suggesting the involvement of a diffusible suppressor of axillary bud development (Beveridge *et al.,* 1994). Studies with the *rms* mutant have revealed a more complex regulatory network involving long-distance novel signals in addition to auxin and cytokinins. In *Arabidopsis*, branchless phenotypes

are observed in plants homozygous for *PINHEAD1* and *REVOLUTA* genes and in the *auxin resistant 3* mutant (Leyser *et al.,* 1996; McConnell *et al.,* 1995, Talbert *et al.,* 1995), while bushy phenotypes characterize the *auxin resistant 1* and *terminal flower 1* mutants (Alvarez *et al.,* 1992; Lincoln *et al.,* 1990). Whether or not hormones are involved in the control of all these phenotypes has not yet been determined but the *altered meristem program* (*amp1*) mutant, which has a decreased apical dominance, is characterized by increased cytokinin levels (Chaudhury *et al.,* 1993). AXR1 acts after lateral bud formation to inhibit lateral bud growth (Stirnberg *et al.,* 1999). Two other genes , *MAX1* and *MAX2*, have also been shown to control lateral branching (Stirnberg *et al.,* 2002).

Many of the genes expressed in the SAM are also expressed in lateral meristems but how the SAM-specific genes become reactivated in these locations is sparsely documented. A gene (*REV*) encoding a homeodomain-zip (leucine zipper) protein was found to have a role in activating meristem activity at lateral positions through activation of the expression of known meristem regulators (Otsuga *et al.,* 2001). A *supershoot* (*sps*) mutant has been isolated, which is characterized by a massive overproliferation of axillary shoots due to an increase in the number of axillary meristems; the *SPS* gene encodes a cytochrome P450 and is involved in modulating hormone levels (Tantikanjana *et al.,* 2001).

2.4.2. Adventitious budding

De novo secondary shoot organogenesis can also occur *in vivo* through adventitious bud formation, reflecting the high potential of homeosis and flexibility in plants (Sattler, 1988). Adventitious buds can arise spontaneously on roots, hypocotyls, stems and leaves (Esau, 1965) following a process of cell dedifferentiation from partially or fully differentiated cells. Dedifferentiation corresponds to a regression of differentiated traits up to the recovery of a proliferative then meristematic condition. Experimentally, it is possible to induce the complete dedifferentiation of very diverse differentiated cells, including epidermis, cortical and leaf parenchyma, pith, and phloem (Buvat, 1989). The spontaneous ectopic formation of buds on leaves (Fig. 8.5; epiphylly) has been extensively described (Dickinson, 1978). Such ectopic bud formation can be induced experimentally by overexpression of the maize *KN1* or of the *Arabidopsis KNAT1* homeobox genes in transgenic tobacco lines (Sinha *et al.,* 1993, Pépin *et al.,* in preparation). High expression of these genes induces bud formation associated with the leaf veins. Whether the natural production of ectopic shoots in other species results from expression of *KN1* homologues in the leaf remains to be determined. The production of adventitious shoots on leaves has also been documented in transgenic tobacco lines that overexpress constitutively (Li *et al.,* 1992; Estruch *et al.,* 1991) or locally (Hewelt *et al.,* 1994) a cytokinin biosynthetic gene. Recent results from several laboratories have suggested reciprocal links between cytokinins and homeobox genes (Frugis *et al.,* 1999; Rupp *et al.,* 1999). In this respect, the role of these parameters in the process of natural or experimental adventitious budding, including *in vitro* bud regeneration, should be determined. Interestingly, endogenous signals appear to modify the response elicited by genes of the *KNOX* family. Ectopic expression of *KNAT1* gene in *Arabidopsis* directs the production of shoots on leaf margins but the vegetative or floral identity of these shoots varies based on the type of leaf from which they are derived (Chuck *et al.,* 1996).

It could be hypothesised that chromatin structure in different tissues may make *KNOX*-binding sites differently accessible and/or that different tissues express some cofactors that modulate the DNA binding or activity of the *KNOX* gene products (Williams, 1998). In addition, there is also evidence suggesting a post-transcriptional regulation of *KNOX* gene expression; regulatory sequences within the *KNOX* gene transcripts could affect mRNA stability so that determinate organs that do not express normally the *KNOX* genes could have a system to degrade the transcripts (Williams, 1998). Ectopic bud formation on leaves has also been reported in a range of *Arabidopsis* transgenic lines overexpressing the *CUC* genes (Takada *et al.,* 2001) which are required for embryonic SAM development. *CUC1* gene would induce adventitious shoots through activation of SAM-related genes such as *STM* (Hibara *et al.,* 2003). Although not involved specifically in SAM determination, the overproduction of the TATA box-binding protein also induces shoot proliferation in transgenic *Arabidopsis* (Li *et al.,* 2001). So, a range of genes is already known to promote adventitious bud formation *in planta* when overexpressed in transgenic lines. Interestingly, explants from these lines also have an increased capacity to regenerate buds *in vitro*, and this could offer new tools for a better control of bud regeneration.

In the transgenic *Arabidopsis* line mentioned above, the *ESR1* (*ENHANCER OF SHOOT REGENERATION 1*) gene which encodes an AP2/ERF (ethylene response factor) transcription factor, confers cytokinin-independent shoot formation when overexpressed in root explants (Banno *et al.,* 2001). However, it has not yet been determined if this gene is involved in SAM identity and/or in cytokinin signaling. Other genes that promote adventitious shoot formation in culture have been identified in *Arabidopsis*. The first such gene is *CYTOKININ INDEPENDENT1* (*CKI1*); it encodes a histidine kinase related to sensory receptors in two-component signaling pathway and has been proposed to be a cytokinin receptor (Kakimoto,1996). Overexpression of *ARABIDOPSIS RESPONSE REGULATOR2* (*ARR2*) also stimulates shoot formation in culture; this gene encodes a B-type response regulator that is thought to be a nuclear activator of cytokinin-responsive genes (Hwang and Sheen, 2001). Overexpression of *CUC1* or *CUC2* also promotes shoot regeneration from calluses in the presence of cytokinins but they do not bypass the requirement for cytokinins (Daimon *et al.,* 2003). The wide variability in the potential to regenerate adventitious buds in tissue culture encountered among species, varieties and cultivars may be explained soon, when a better knowledge about the regulation and interrelationships of genes involved in lateral and adventitious SAM establishment will be acquired.

3. REPRODUCTIVE MORPHOGENESIS

While germ and somatic cell lines diverge very early in embryonic animal development, there is no germ line at all in plants and the decision for germ cell differentiation is put off usually until very late in development. The transition to flowering occurs in shoot apical meristems, which are reprogrammed to make inflorescence or floral organs rather than vegetative organs upon receiving appropriate environmental or developmental signals (Howell, 1998; Lyndon, 1990; Bernier *et al.,* 1993). This change in determination of the SAM cells can be split into several successive steps. The first of these is the induction of flowering, i.e. the decision to switch from the vegetative to the generative phase. The second step is evocation, when the transition from the vegetative to the reproductive organization of the SAM takes place. The third step corresponds to the formation of inflorescence and flowers from the rearranged SAM. Later, the flower organs gain their characteristic forms and functions so that pollination and fertilization can take place. At the level of the whole plant, the SAM reprogramming towards flowering is probably also accompanied by the acquisition of new properties by the differentiated organs as shown by tissue culture experiments. Leaf stem or root explants excised from vegetative plants usually regenerate vegetative buds while explants excised from flowering plants may regenerate reproductive buds (Chouard and Aghion, 1961; Paulet and Nitsch, 1964; Brossard, 1979; Chuck *et al.,* 1996).

3.1. STRUCTURAL REARRANGEMENTS OF THE SAM DURING THE REPRODUCTIVE PHASE

An increase in size and in growth rate of the SAM is characteristic of the commitment from making leaves to making flowers (Fig. 8.6). This phase change is usually accompanied by the loss of the cytophysiological zonation of the SAM due to the activation of cells of the central zone and in a more dome-shaped outline (Nougarède, 1967). The modified SAM can generate either a unique flower or an inflorescence meristem that will give rise to several flower meristems. The L1 layer contributes to the epidermis, the stigma, part of the transmitting tract and the integument of the ovules (endothelium) while the L2 and L3 layers contribute to different degrees, to the parenchyma and other internal tissues (Jenik and Irish, 2000). Germ cells usually derive from the L2 layer (Stewart, 1978) but the anticlinal divisions of the L1 and L2 layers and the divisions in all planes in the L3 layer must be coordinated in the inflorescence meristem and in the flower primordia. The role of floral homeotic genes *APETALA 3* and *AGAMOUS 1* (*AG1*) has been studied by clonal analysis. It was concluded that *AG1* is required to maintain the layered structure of the floral meristem prior to organ initiation (Jenik and Irish, 2000).

Evidence for signaling between cell layers was provided in *Camellia* graft chimeras: the *C. sasanqua* stock has single flowers with a single whorl of petals and normal stamens and carpels; the *C. japonica* scion is a double-flowered form with sepals and many whorls of petals but no stamens or carpels. The chimera consists of an L1 layer from *C. sasanqua* and L2 and L3 layers from *C. japonica* and its flowers resemble those of *C. sasanqua* for the epidermal characteristics, but have also stamens and carpels, suggesting that a signal supplied in the L1 layer can move between cell layers to correct the developmental defect in the L2 and L3 layers

(Stewart *et al.,* 1972). As for vegetative SAMs, the possibility for movement of transcription factors between cells was also observed following studies of the localization of the snapdragon GLOBOSA and DEFICIENS proteins, both transcription factors, suggesting that movement of transcription factors may be a generalized phenomenon *in planta*. Much work remains to be done to detect such movements in systems grown *in vitro* and to demonstrate their involvement in regeneration processes under the influence of growth substances.

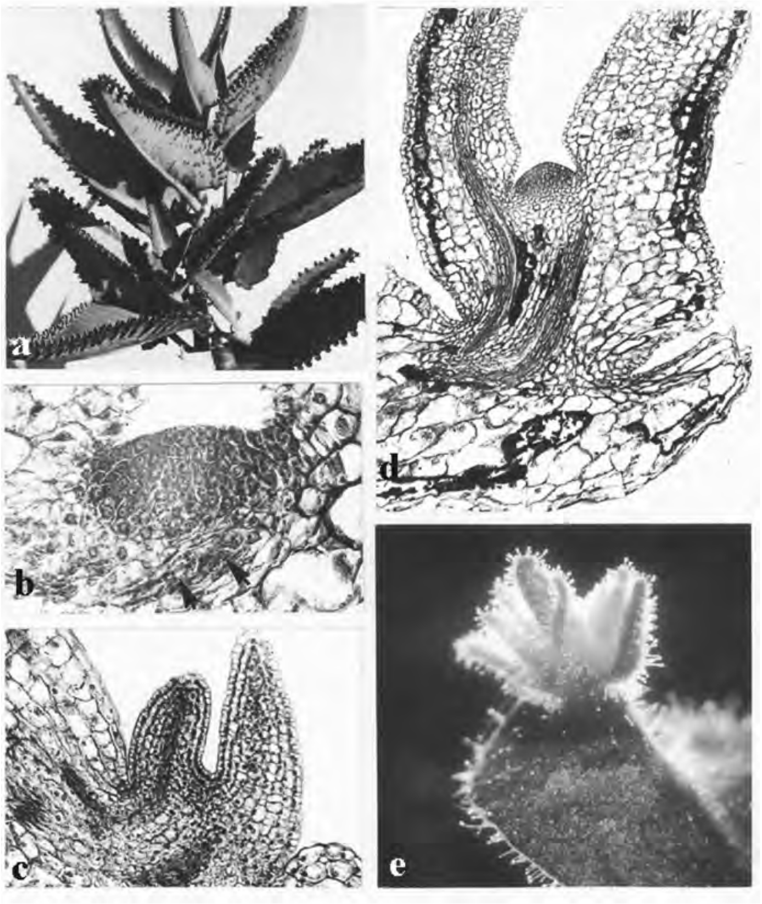

Fig. 8.5 – Adventitious budding, natural on *Bryophyllum daigremontianum* leaves under 16h photoperiod (a: morphology; b to d: three stages of bud establishment) and experimentally induced through transfer of a cytokinin-encoding *IPT* gene at the tip of a transgenic tobacco leaf (e).

Under inductive conditions, the switch to the flowering stage is first marked by a prefloral phase during which the SAM becomes enlarged and creates abundant cell material. The meristem changes in shape, increases in size and loses both its zonated structure and the plastochronic function. The prefloral phase is followed by a reproductive phase during which flower primordia organize and diversification occurs depending on the particular species. These changes have been reported following detailed examinations of the ontogenic development of numerous flowers and inflorescences (Lance, 1957). Some species such as *Sinapis alba* (Bernier *et al.,* 1981) and *Arabidopsis thaliana* (Besnard-Wibaut, 1975) recover zonation during the prefloral phase.

Under non-inductive conditions, for example when long-day or short-day species are submitted to a photoperiod that is consistently unfavourable for entering the prefloral phase, the SAM continues to produce leaves. However, it is no longer structurally vegetative and acquires a new type of

structure, with a partial accentuation of the meristematic characteristics of the central cells, a periclinal stratification of the corpus and of the rib meristem. These new characteristics define the "intermediate phase" (Lance, 1957). During this phase, the orientation of the cell divisions typical of the normal ontogeny is modified, the L1 layer displaying a tighter control than others. This suggests that there must be genetic programs that control the patterns of cell division in a layer-specific manner.

3.2. CONTROL OF FLOWERING TIME

Adult SAMs become competent for flowering dependent on a range of environmental (day-length, temperature) and internal (developmental state) signals according to the species. In *Arabidopsis*, which is regulated by both long days and winter cold (vernalization), early and late flowering mutants have been isolated, allowing the identification of key genes controlling flowering time (Araki and Komeda, 1993; Araki, 2001). Genetic analysis of the control of flowering has identified four major floral promotion pathways (Fig. 8.7). The photoperiod and vernalization pathways mediate the response to environmental signals, whereas the autonomous and gibberellin pathways appear to act independently of these signals. In the photoperiod pathway are the *CONSTANS* (*CO*) and *FT* genes because mutations in these genes delay flowering in long days but not in short days. *CO* encodes a putative transcription factor whilst *FT* encodes a protein with similarity to RKIP proteins (Hepworth *et al.,* 2002). The vernalization pathway appears to be controlled by the synergistic interaction of *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*) genes. Both the autonomous pathway and vernalization regulate negatively *FLC* which encodes a MADS domain-containing transcription factor that inhibits flowering in biennials unless they have been exposed to the cold winter (Michaels *et al.,* 2005). This floral repressor would be up-regulated by *FCA* gene and has a down-regulated effect the *SOC 1/AGL 20*, factors involved in the floral transition.

The inactivation of the floral repressor has been suggested to lead to the activation of the floral meristem identity genes such as *LEAFY* (*LFY*) or its analogue *FLORICAULA*(*FLO*) in snapdragon, and *APETALA1* (*AP1*), which specify the floral meristem identity of early lateral primordia produced by the SAM. The *EMF* (*EMBRYONIC FLOWERING*) genes appear also to play a role in repressing flowering because mutants of these genes flower without a preceding vegetative phase. This reinforces the hypothesis that flowering is a default pathway. The progression from vegetative to flowering phases appears to be regulated by multiple and complex genetic pathways (Fig. 8.7) to which must be added epigenetic effects. The similarity of some genes involved in repression of flowering, with animal genes of the Polycomb group (PcG) involved in homeobox gene expression, has suggested that they could act similarly. The PcG proteins act in altering chromatin structure, repressing reproductive development. There is also evidence that chromatin modelling can affect flowering by changing methylation patterns (Mouradov *et al.,* 2002) and that miRNAs control genes involved in flowering time control *via* a translational repression mechanism (Aukerman and Sakai, 2003).

3.3. DETERMINATION OF FLORAL AND INFLORESCENCE MERISTEM IDENTITY

In addition to their role in promoting the floral transition, the *LFY/ FLO* genes play also a role in later stages (Fig. 8.7). The flower or inflorescence identity of the SAM after flowering induction is controlled by the opposite action of two sets of genes. The *lfy* mutation results in the production of inflorescences in place of flowers, while the *tfl* (*terminal flower*) mutation results in the conversion of the inflorescence meristem into a flower. The *TFL* gene is therefore required to promote inflorescence identity or suppress floral meristem identity in the inflorescence meristem (Coen and Carpenter, 1993). *APETALA1* (*AP1*) gene is also necessary to specify floral meristem identity and functions downstream of *LFY*, itself required for the proper expression of floral organ identity genes during the late stage of the reproductive phase (Mandel and Yanofsky, 1995a and b).

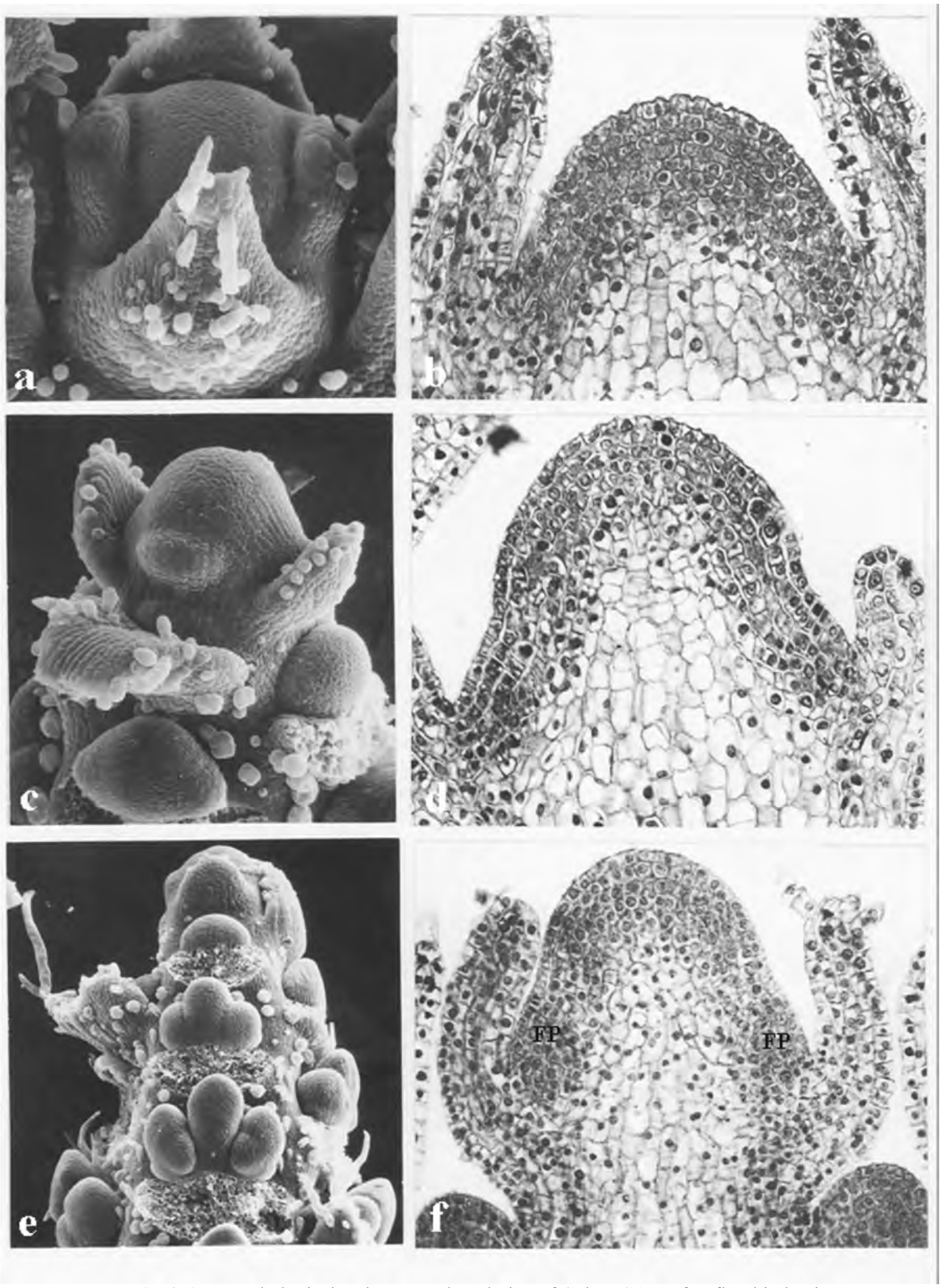

Fig. 8.6 – Morphological and structural evolution of Coleus SAM after floral induction.
a,c,e: scanning election microscopy; b,d,f: corresponding longitudinal sections. At the prefloral phase (a,b), the SAM loses the cytophysiological zonation and becomes entirely meristematic, then, at the floral phase, the meristematic cells organize the floral primordia acropetally, building the inflorescence progressively (c, d then e,f). (courtesy of R. Saint-Côme, 1985).

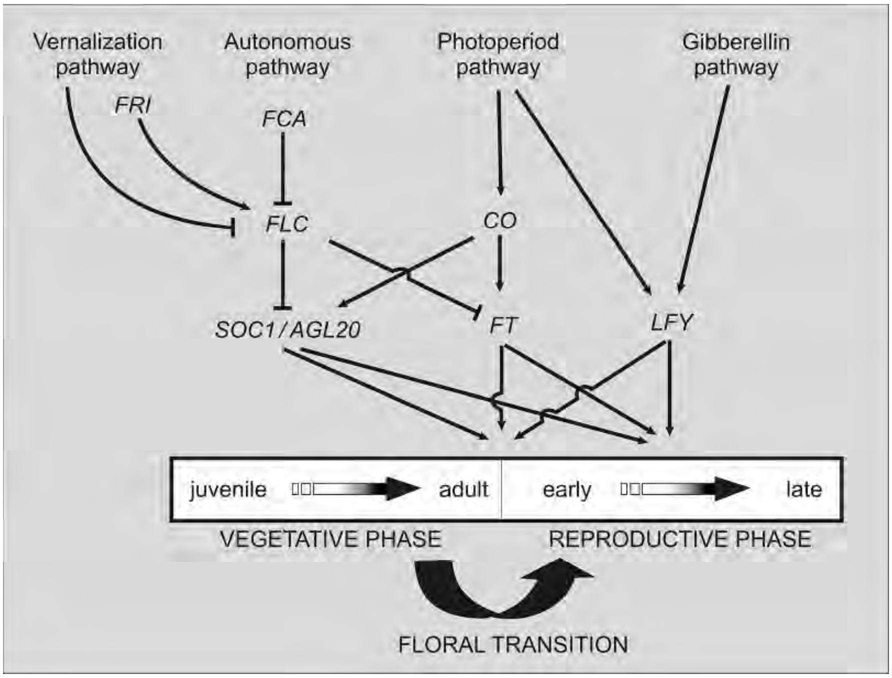

Fig. 8.7 – Genetic pathways that enable floral transition.
Plants integrate multiple cues during the switch from the vegetative to the reproductive phase including the vernalization, autonomous, photoperiod and gibberellin pathways. Vernalization and the autonomous pathways promote floral transition by reducing the levels of the floral repressor FLC. The photoperiod pathways (under long days) act *via* a transcription factor, CO. The gibberellin pathway has an essential role in short-day conditions. These four pathways are integrated at the transcriptional regulation of two "flowering-time" genes, *FT* and *SOC*. *FRI* = *FRIGIDA*; *FCA* = gene controlling the flowering autonomous pathway; *FLC* = *FLOWERIN*G *LOCUS C*; *CO* = *CONSTANS*; *FT* = *FLOWERING LOCUS T*; *LFY* = *LEAFY*; *SOC* = suppressor of overexpression of *CONSTANS*. (redrawn and modified from Araki, 2001, by P. Rech, University P. M. Curie).

3.4. DETERMINATION OF FLORAL ORGAN IDENTITY

Floral organs form in concentric rings, called whorls. Sepals form first, followed by petals, then by stamens, and finally by carpels. One of the best understood parameters of floral meristem determination concerns the floral homeotic genes that are responsible for specifying organ identities in the flowers (Fig. 8.8). In the fruit fly *Drosophila*, homeotic mutations led to the identification of a set of homeotic genes encoding transcription factors involved in the determination of the location at which specific organs develop. In plants, such genes have been identified and belong to a class of related genes known as MADS box genes provided by a conserved DNA-binding domain, the acronym "MADS" being derived from the first letter of the first five members of this class of genes, including the plant floral organ identity genes *AGAMOUS* and *DEFICIENS*.

There are three main classes of homeotic genes involved in flower organ identity, each class affects the identity of two adjacent whorls of organs. These classes of genes have been designated A, B and C. The class A genes are *APETALA 1* and *2* (*AP1* and *AP2*) and their mutations cause the sepals and petals to form carpels and stamens, respectively. Two genes, *APETALA 3* (*AP3*) and *PISTILLATA* (*PI*) belong to class B and mutations in either *AP3* or *PI* cause petals and anthers to form sepals and carpels, respectively. The class C gene is called *AGAMOUS* (*AG*) and *AG* mutations cause the stamens and carpels to form petals and sepals, respectively. A model named ABC (Fig. 8.8) was proposed where the overlapping action of these genes would specify organ identity (Coen and Meyerowitz, 1991). The model also predicts that A and C activities are mutually exclusive. *LFY* plays a pivotal role in regulating floral organ identity as it regulates the expression of A, B and C class genes (Parcy *et al.,* 1998). In addition, ectopic expression studies indicate that the ABC genes are not sufficient to convert vegetative leaves into flower organs, so a new class of floral identity MADS box genes (*SEPALATA*) was recently described (Pelaz *et al.,* 2001). In the revised model, *SEP* genes are referred to as E class while a D class specifying ovule identity completes the model (Colombo *et al.,* 1995). The

majority of ABCDE genes encodes MADS transcription factors but higher orders of complexity involving miRNAs are now expected (Jack, 2004).

In addition to *Arabidopsis* and *Antirrhinum*, the molecular genetics of flower development is now being extended to other species (Kyozuka *et al.,* 2000) and the results suggest that the basic mechanisms have been conserved in evolution but further insights are still necessary to study genes that have no obvious counterparts among species. It is still unclear whether the products of the flower organ identity genes act directly on the effector genes for central cell function or whether additional steps are involved. Interestingly, the MADS-box gene phylogenetic tree reveals that gymnosperms lack the *A*-function gene, which is likely the reason why these plants do not have petals and sepals (Shindo *et al.,* 1999).

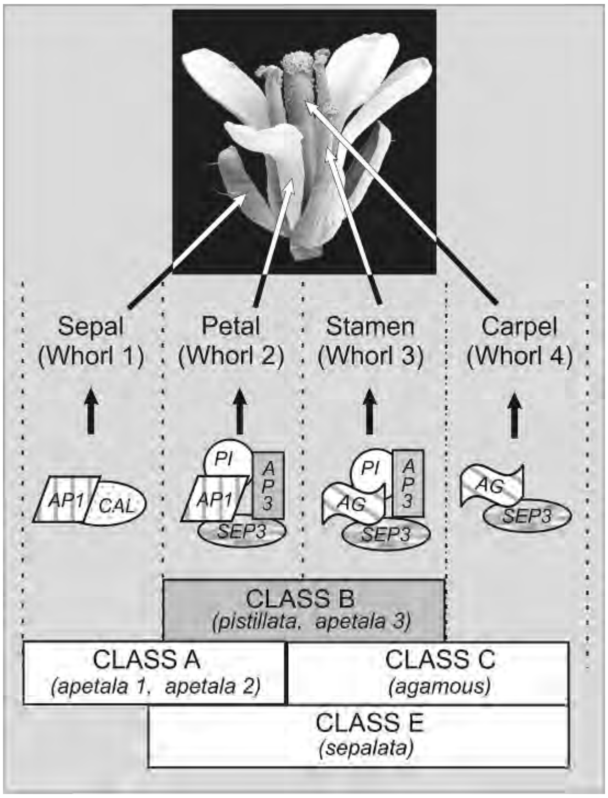

Fig. 8.8 Genes controlling floral organ specification according to the revised ABC model.
The *SEP* genes play a crucial role in addition to the *A*, *B* and *C* genes in providing transactivation activity and serving as a scaffold between B and C proteins. *AP – APETALA, AG – AGAMOUS*, *PT – PISTILLATA*, *SEP – SEPALATA*
(redrawn and completed from Goto *et al.,* 2001, by P. Rech, University P. M. Curie)

4. ZYGOTIC EMBRYOGENESIS

4.1. PATTERN FORMATION FROM THE ZYGOTE TO THE MATURE EMBRYO

Zygotic embryos develop within the embryo sac from the fertilized ovules on mother plants according to an orderly pattern. Embryogenesis in all flowering plants is more or less similar, even though the seedlings of monocotyledonous and dicotyledonous plants differ in their appearance and construction. The major difference between the two lies in the presence of one or two cotyledons flanking the future SAM. Double fertilization generates the diploid zygote and the triploid endosperm. The endosperm undergoes a series of developmental events and provides nutrients to the developing embryo (Lopes and Larkin, 1993) while the zygote divides several times to give rise to the embryo (Fig. 8.9). The generation of the embryo from a single cell requires the spatially coordinated acquisition of numerous cell identities.

Embryogenesis can be divided into three phases. The first phase is one of morphogenesis during which the polar axis and the radial patterning of the plant body is defined with the specification of the shoot and root apex domains, and the embryonic tissues and organs are formed. The second phase is one of embryo maturation; it is characterized by the accumulation of storage material. During the final phase, the embryo becomes desiccated and enters a period of developmental arrest. At the beginning the zygote establishes an early polarity, with unequal distribution of cytoplasm and vacuoles (Schulz and Jensen, 1968), and elongates in the micropylar-chalazal axis, establishing the apical-basal axis of the future embryo. This coincides with a reorientation of microtubules to transverse cortical arrays (Webb and Gunning, 1991). The first division is an unequal cell division resulting in two cells of different developmental fates: a small and sparsely vacuolated apical cell that gives rise to the bulk of the embryo proper and a larger and vacuolated basal cell that develops into the hypophysis and the suspensor. Several embryonic types, according to more or less variable cleavage patterns, have been reviewed by Mordhorst *et al.* (1997) but most of these types develop through the same stereotyped morphological stages of globular, heart and torpedo (Pollock and Jensen, 1964).

In *Arabidopsis*, starting from the point of fertilization, the development of a mature embryo takes 9 days at 25 °C then the process of maturation requires two additional days. In this time, the embryo grows into approximately 20,000 cells and reaches a diameter of 500 µm, the initial zygote being only 20 µm in diameter (Jürgens, 1998). After the first asymmetric division of the zygote, the apical cell undergoes two longitudinal divisions to produce a 4-celled embryo proper. A transverse division follows to produce two tiers of cells in an octant stage. The next division of the octant stage is parallel to the embryo surface, establishing the first histologically detectable tissue, the protoderm (i.e. the future epidermis). The delineation of the protoderm establishes the globular stage embryo in which increases in size and cell number occur by anticlinal divisions of the protoderm, and longitudinal then transverse divisions of inner cells. At this time, the suspensor has reached maximal cell number and begins to senesce soon after, and the embryo proper is axialized but not yet asymmetrically polarized in the apico-basal dimension. This is achieved rapidly by successive oriented cell divisions that generate narrow cells in the center of the lower tier of the embryo. These cells generate longitudinal cell files corresponding to the future central cylinder. Soon afterwards, cell divisions occur in specific places of the lateral regions of the upper part of the globular embryo, resulting in two cotyledon primordia and the establishment of a heart-shaped morphology. The switch from radial to bilateral symmetry introduces delineation between the embryonic axis and the cotyledons. Following their formation, both the axis and the cotyledons elongate and give rise to the torpedo stage embryo. Domains corresponding to the future shoot and root meristems can be distinguished from the beginning of the heart stage. In the basal pole of the embryo, the quiescent centre and the initial cells of the root cap are derived from the hypophysis, while the initials of the root meristem derive from the lower tier of the octant pro-embryo (Scheres *et al.,* 1994).

During the third phase (maturation), storage macromolecules accumulate in most of the cells, inducing a rapid increase in mass and size of the embryos and, in late embryogenesis, embryos also acquire the ability to withstand desiccation and enter metabolic quiescence until they encounter favourable conditions for germination. During the quiescent state, cells of the future apical meristems have been found to be arrested in the G_1 state of the cell cycle (Rembur, 1972) while cells of the primary body of the embryo can display a more or less pronounced polyploidy (Nagl, 1979).

4.2. GENES CONTROLLING EMBRYOGENESIS

Embryogenesis in higher plants is a process dependent on coordination of specific genetic programs and proper communication between different parts of the developing seed. A precise order of events ensures the correct positioning of embryonic organs, i.e. the shoot and root meristems, the cotyledons and the hypocotyls (Berleth and Chatfield, 2002). A large number of embryonic mutants have been identified in *Arabidopsis* and in maize, including mutants altered in their pattern formation (Mayer *et al.,* 1993; Berleth and Jürgens, 1993), meristem establishment (Barton and Poetig, 1993) and maturation programs (Keith *et al.,* 1994; Meinke, 1992). Recently, a systematic expression analysis of a family of homeodomain-containing transcription factors that are homologous to *WUSCHEL* (*WUS*) has demonstrated that plant embryos are partitioned early into regions marked by

the expression of these factors, as in animal embryos (Haecker *et al.,* 2004).

Among the genes identified as involved in the apico-basal patterning, it was shown that mutations in the three genes *MONOPTEROS* (*MP*), *FACKEL* (*FK*) and *GURKE* (*GK*) delete early specific embryo parts. *Monopteros* homozygotes lack both hypocotyl and roots. This gene seems to be required for the organization of the basal regions of the embryo. In *fk* embryos, the central region at the globular-stage is abnormal and the hypocotyl is missing such that the cotyledons are directly attached to the root; in *gk* embryos, the apical region is abnormal and embryos lack a shoot meristem and cotyledons (Jürgens, 1998) and the *GURKE* gene encodes an acetyl-CoA carboxylase which is required for partitioning the embryo into three subregions (Kajiwara *et al.,* 2004). The *GNOM* (*GN*) gene controls the asymmetric division of the zygote and the corresponding mutant has alterations in the entire apical-basal pattern; *GN* encodes a endosomal regulator of vesicle budding and has been shown to regulate auxin efflux (Geldner *et al.,* 2003). *HOBBIT* (*HBT*) drives the normal division of the hypophysis in the globular embryo and is required for formation of the root meristem; *HBT* encodes a CDC27 homologue required for cell division and cell differentiation in the meristems. *Hobbit* mutants have no active root meristem and lack a quiescent center and root cap columella, they also show an accumulation of the AXR3/IAA17 repressor of auxin responses (Blilou *et al.,* 2002). *BODENLOS* (*BDL*) is involved in the formation of the embryo axis through a defective response to auxin (Hamman *et al.,* 1999). Mutations in the *FACKEL* (*FK*) gene, which encodes a sterol C-14 reductase, result in seedlings with very reduced hypocotyls (Schrick *et al.,* 2000), indicating that defects in sterol synthesis also impinge on the embryo axis formation.

Concerning the radial patterning, *SCARECROW* (*SCR*) regulates separation between endodermal and cortical layers (Jürgens, 1998). The *SCR* gene, which encodes a putative transcription factor, may confer endodermal cell identity. The endodermis is also absent in *short-root* (*shr*) embryo mutants. The *SHR* gene is a member of the GRAS family of putative transcription factors; it is involved in correct radial patterning through SHR intercellular movement from the stele to the target initial cells of the endodermis (Sena *et al.,* 2004). Radial pattern formation starts in the 8-celled embryo and involves two choices of oriented cell division: periclinal (radial) which gives tissue layers and anticlinal which increases the number of cells in a given layer. The *KNOLLE* (*KN*) gene affects cell division; the *kn* mutant embryo displays incomplete separation of the daughter cells and the epidermis is not separated from the inner cells by periclinal divisions. Specification of the protoderm appears very early in the octant-stage embryo with the expression of the *Arabidopsis thaliana meristem layer 1* (*AtML1*) which encodes a homeodomain-containing putative transcription factor (Fig. 8.10). This gene is expressed in the apical daughter cell of zygote and in all the cells of the globular embryo during the early stages, then the expression becomes restricted to the epidermis, suggesting the existence of some outside-in signaling (Sessions *et al.,* 1999). The protoderm also expresses the *LIPID TRANSFER PROTEIN* (*LTP*) gene at an early stage, and this continues to be expressed in the derivatives of the epidermal primordium throughout development (Thoma *et al.,* 1994). Both genes are therefore tissue-specifically expressed and can be used as good markers of the epidermis.

Another class of embryo mutants has been described as shape mutants, as they produced seedlings with altered phenotypes such as growth retardation (*mickey*) or extreme compression along the axis of the body organs (*fass*). A detailed analysis of *fs* mutants revealed that all pattern elements originate at the appropriate location, although cell division planes were altered with a lack of microtubule preprophase bands (Traas *et al.,* 1995). Specific genes are also required for the establishment of the future shoot and root meristems which are nearly inactive in mature embryos. As previously explained, the *SHOOT MERISTEMLESS* (*STM*) gene is expressed in the cells that will become the SAM and homozygous *stm* mutants do not form any SAM (Lincoln *et al.,* 1994). *STM* expression is observed in one or two cells at the apical end of the mid-globular embryo. The *STM* gene has been cloned and shown to be a member of the KNOTTED class of homeoprotein genes (Table 1; Long *et al.,* 1996). Expression of *KNOTTED1* marks SAM formation during maize embryogenesis (Smith *et al.,* 1995). At the heart stage, expression is confined to a few cells between the cotyledons. This gene is also required for the maintenance of the undifferentiated state of the SAM central zone in the adult plant. *STM* is turned off as cells are recruited towards the formation of lateral organs, the lateral founder cells being characterized by the expression of the MYB domain transcription factor *ASYMMETRIC LEAVES 1* (*AS1*)(Byrne *et al.,* 2000). The *zwille* mutation also

disrupts SAM establishment during embryogenesis but does not interfere with post-embryonic SAM formation (Jürgens *et al.,* 1994). The *CUP-SHAPED COTYLEDONS* (*CUC*) genes may also participate in embryonic SAM establishment as they are expressed in the same domain as *STM* during embryo formation (Vroemen *et al.,* 2003) and they interact with STM (Aida *et al.,* 1999). The *WUSCHEL* (*WUS*) gene appears as an early regulator of stem cell identity; *WUS* transcripts are first detected in the 16-32 cell embryos in all the subepidermal cells of the apical domain, then they become gradually restricted to more central and deeper cells at the base of the future SAM (Fig. 8.10; Mayer *et al.,* 1998).

The maturation program in late embryogenesis involves several specialized gene sets such as those encoding storage proteins and late embryo abundant (LEA) proteins that are activated transcriptionally during maturation then repressed before dormancy (Goldberg *et al.,* 1994). A mutant gene class that causes defects in cotyledon, differentiation and maturation specific events, *lec* mutations transform cotyledons into leaf-like structures and the *lec* embryos fail to survive desiccation, do not accumulate storage proteins and germinate precociously. The *LEC* gene encodes a transcription factor sharing similarities with the *VIVIPAROUS1/ABA INSENTITIVE3* genes (Stone *et al.,* 2001). Cotyledon identity is also altered in the *fusca 3* (*fus3*) mutants. Other *Arabidopsis* mutants are *abscisic acid insensitive* (*abi*) due to alteration in loci involved in abscisic acid synthesis and perception; the resulting phenotypes lacks desiccation tolerance, storage accumulation and embryo dormancy. Similarly, the *viviparous 1* (*vp1*) maize mutant embryos are also hyposensitive to ABA. All these genes have now been cloned and correspond to transcription factors. The identification of protein that interacts with these factors and their functional analysis are in progress and it is clear that these genes will be of considerable interest in the manipulation of somatic embryogenesis.

4.3. PHYTOHORMONES AND EMBRYO FORMATION

The participation of phytohormones in seed development has been frequently reported and hormone analysis has revealed high levels of both auxin and cytokinins in developing seeds, abscisic acid being more particularly involved in seed maturation (Morris, 1997). Auxin has been implicated as at least one of the signals involved in apical-basal patterning of the embryo (Fig. 8.10). Inhibitors of auxin transport block both mono- and dicotyledonous embryos at the transition globular-heart stages and prevent cotyledon separation (Liu *et al.,* 1993; Fischer and Neuhaus, 1996). The resulting phenotypes, with fused cotyledons, are reminiscent of the cup-shaped androgenetic embryos described in the past as being formed in the presence of high auxin concentration (Nitsch, 1969). They also resemble the *Arabidopsis pm1-1* mutant embryos (Liu *et al.,* 1993), which are defective in auxin efflux carriers. An auxin gradient is involved in the apical-basal axis formation (Friml *et al.,* 2003). The *monopteros* and *gnom* mutants are characterized by alterations in both embryo axis formation and vascular development and the *MONOPTEROS* (*ARF5*) and *GNOM* (*GN*) gene encode an auxin response factor (Hardtke and Berleth, 1998) and a guanine nucleotide exchange factor required for the correct localization of the auxin efflux carrier PIN1 (Grebe *et al.,* 2000). Auxin polar transport appears therefore necessary to determine the apical-basal axis formation (Steinmann *et al.,* 1999). Other evidence of auxin signaling during embryogenesis includes the finding that the putative auxin receptor ABP1 is required for organized cell elongation and transition to bilateral symmetry (Chen *et al.,* 2001).

In addition, mutations in *CUC1* and *CUC2* genes result in fused cup-shaped cotyledons, suggesting that *CUC1* and *CUC2* are involved in organ separation (Aida *et al.,* 1997) but the possible control of these genes by auxin has not yet been studied. Lack of auxin polar transport is also responsible for the induction of poly-embryos (Fischer *et al.,* 1997). Suspensor cells may have the potential to regenerate supernumerary embryos. This was demonstrated with the *Arabidopsis raspberry* and *twin* mutants but the nature of the muted genes is still unknown. One possibility is that the embryo proper transmits inhibitory signals to the suspensor that suppress the embryonic pathway; alternatively, the balance of growth regulators in the entire embryo may maintain the development states of both the embryo proper and the suspensor, and alteration of their balance may cause the suspension to undergo an embryogenic pathway (Goldberg *et al.,* 1994).

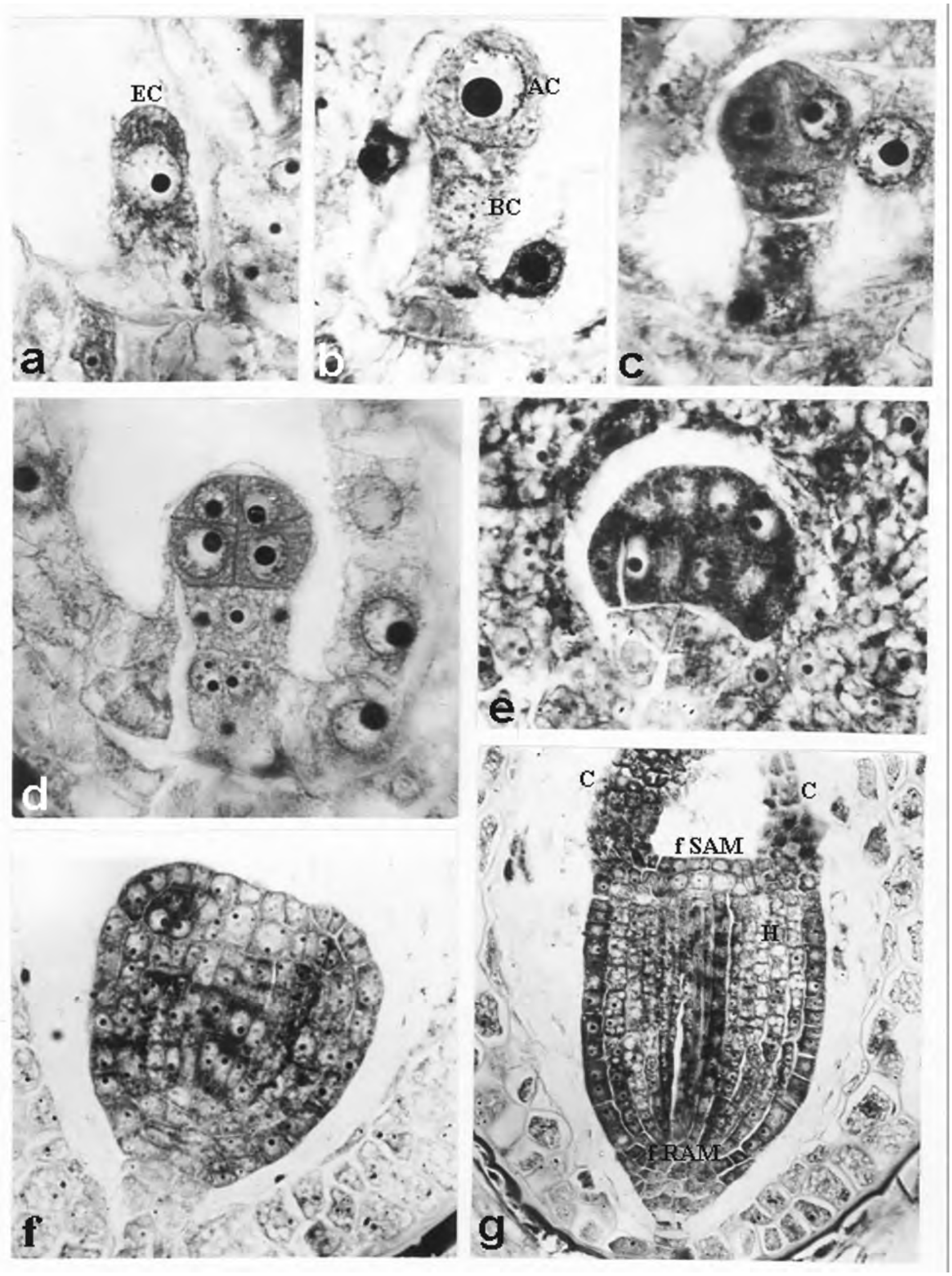

Fig. 8.9 – Sequence of *Capsella* embryogenesis from the egg cell (a) to the final embryo (g).
b: 2 cell–stage; c: 4 cell-stage; d: 8 cell-stage; e: globular embryo; f: heart-shaped embryo. AC: apical cell, BC: basal cell, fSAM: future shoot apical meritem; fRAM: future root apical meristem; C: cotyledons; H: hypocotyl (courtesy of P. Rondet, 1965).

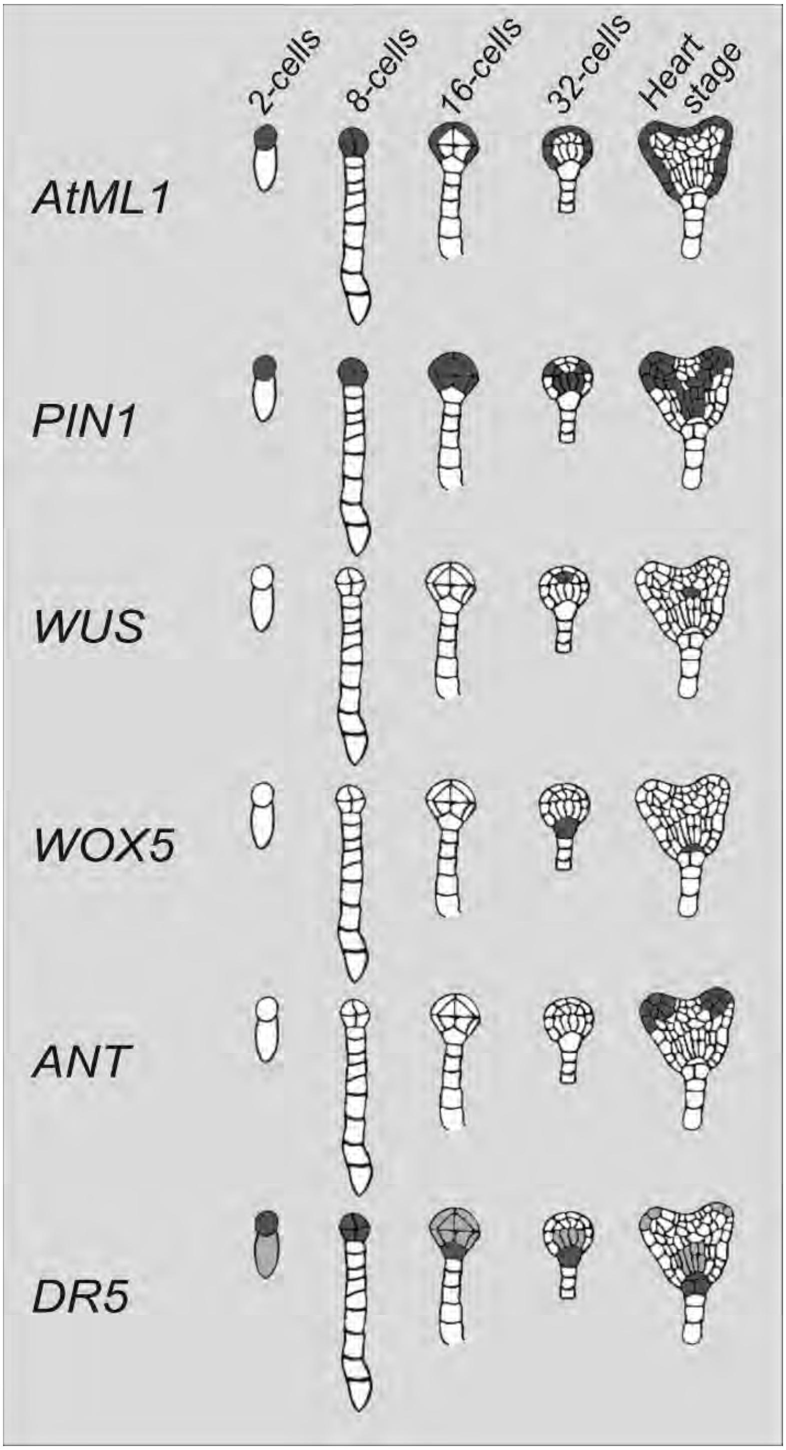

Fig. 8.10 – Patterns of gene expression during zygotic embryogenesis.
Sites of expression of genes involved in L1 layer specification (*ATML*1), auxin efflux (*PIN*1), SAM organization centre identity (*WUS*), RAM quiescent centre identity (*WOX*5), lateral organ formation (*ANT*) and auxin presence (*DR*5) are shaded.
(redrawn and modified from Weijers and Jürgens, 2005, by P. Rech, University P. M. Curie).

Whether or not cytokinins are also involved in embryo patterning is not yet established but local expressions of the *ipt* gene which encodes cytokinin biosynthesis promotes twin embryos (Offringa, personal communication), suggesting that this hormone could act together with auxin in embryogenesis as in other developmental pathways. Loss of function mutations in the *CYTOKININ RESPONSE 1/WOODEN LEG* (*CRE1/WOL*) gene result in reduced cell divisions in the root meristem and a lack of phloem formation in the hypocotyls and roots (Mähönen *et al.,* 2000); this gene encodes a cytokinin receptor (Inoue *et al.,* 2001), supporting the implication of cytokinins in cell division and cell differentiation within the vascular cylinder.

4.4. ASEXUAL EMBRYOGENESIS *IN PLANTA*

The ability to form embryos that do not originate from a fertilized egg cell exists in some species. It may occur naturally in *Malaxis*, where somatic embryos form spontaneously on the leaf tips (Taylor, 1967) or ovules by apomictic processes (Koltunow, 1993). Under *in vitro* conditions, somatic embryogenesis is now a quite widespread phenomenon, as reviewed in Chapter 9. Curiously, genes that have been listed for their involvement in zygotic embryogenesis have been studied little for a role in somatic embryogenesis. It has only been reported that somatic embryogenesis is facilitated starting from immature zygotic embryos of mutants such as *clv* that have an increased population of non-committed SAM cells (Mordhorst *et al.,* 1998).

5. ROOT MORPHOGENESIS

During embryogenesis, opposite to the SAM pole, a root pole is differentiated with a future root apical meristem. There is no reliable report of direct interconversion of these meristems during normal development. However, differentiated cells of both shoot and root organs from many species may regenerate meristems of either type, both *in planta* and in tissue culture. Phylogenetically, roots are more recent organs than stems and leaves and their emergence has constituted one of the important and determining parameters for the growth of plants on land. The finding that gene cassettes are providing some similar developmental functions in shoot and root should not come as a surprise to plant evolutionary biologists for whom it has always appeared likely that the root meristem evolved from an ancestral shoot mersitem (Benfey, 1999a). Nevertheless, a range of processes appear to be only root-specific.

5.1. STRUCTURAL ORGANIZATION OF THE ROOT APICAL MERISTEM

5.1.1. General Organization

Angiosperm roots have a rather stereotypic anatomy with regular cylindrical layers representing the tissue types of the root (epidermis, cortex, endodermis, pericycle) which in turn enclose a central vascular cylinder. The root tip (Fig. 8.11a, b) consists of two groups of cells: a cap at the extreme apex, and the root proper which the cap surmounts. Both the cap and the root proper may be regarded as being composed of columns (or files) of cells. Longitudinal growth of the root is mediated by the root apical meristem (RAM). Unlike the SAM, the RAM elaborates a root cap that protects the root tip as it pushes through the soil. The root cap is made up of cells that continuously slough off as the root grows. Root cap cells (statocytes) also contain starch grains (statoliths) which are the sensors of the gravity stimulus.

In Angiosperms provided with a closed RAM type (*Arabidopsis*, maize) the cell files that make up the cylindral layers converge into the root apex. In species with open RAM type (pea, Java bean), the cell files do not converge but appear to constrict, then extend into the root cap (Doerner, 1998). Each cell file originates from initial cells located at its apical end and previously named histogens (Hanstein, 1870). In monocotyledonous plants, the dermatogen establishes only the external layer and the root cap has a separated origin; in dicotyledonous plants, the root cap is in continuity with the dermatogen. Roots develop by the extension of continuous tissue layers in which occurs channeled signaling (Scheres and Berleth, 1998). In addition to organization in cell files, the RAM displays also a zonation, as revealed by histoautoradiography and microspectrophotometry (Clowes, 1961, 1965). The various zones are characterized by a more or less intense mitotic activity. The quiescent center, now renamed the RAM organizing center, has reduced mitotic activity with most cells arrested in the G1 phase of the cell cycle. This quiescent center controls stem cell differentiation. The proliferating zones, including the initials, are located above and below the quiescent center, the first one giving rise to the cortex and

central cylinder and the other to the cap. The inherent organizing capacity of the quiescent center for root development was demonstrated by *in vitro* culture of isolated quiescent centers of corn that were able to form whole roots directly without intermediate callus formation (Feldman and Torrey, 1975). Beyond the RAM, cells elongate (elongation zone), then differentiate.

5.1.2. Origin of the primary root meristem

Very detailed studies of cell fates have been published for the embryonic origin of the *Arabidopsis* RAM (Dolan *et al.,* 1993, 1994; Scheres *et al.,* 1994). The RAM comes from two clonally distinct cell populations: the upper tier of stem cells is derived from the apical daughter cell of the zygote whereas the quiescent center and the lower tier of stem cells originate, via the hypophysis, from the basal daughter cell of the zygote. Cell lineage analysis was performed with transgenic plants expressing the *35S:Ac:GUS* construct, the excision of the *Ac* transposable element activating the *GUS* expression in sectors of the RAM that serve as cell lineage markers. This approach confirmed cell fates predicted by the anatomical analysis. Common origin for external layer and lateral root cap cells was demonstrated as well as for cortical and endodermal cells. Despite the apparent rigid organization, the root tip was also demonstrated as capable of flexibility as revealed by laser cell ablation experiments (Van den Berg *et al.,* 1995).

5.2. GENES INVOLVED IN PRIMARY ROOT IDENTITY AND PATTERNING

5.2.1. Control of RAM, quiescent center and initials identity

Root development initiates with root specification. The *HBT* (*HOBBIT*), *BDL* (*BODENLOS*) and *AXR6* (*AUXIN RESISTANT 6*) genes are required during embryogenesis and appear to have a pivotal role in root formation (Weigel and Meyerowitz, 1994; Willemsen *et al.,* 1998). Mutations in these three genes give seedlings that lack a primary root but are capable of forming roots post-embryonically. These genes appear to mediate auxin-dependant processes. *Arabidopsis* mutants homozygous for a mutation in the *ROOT MERISTEMLESS* (*RML1*) gene are unable to establish an active root meristem and are glutathiol depleted; this mutation abolishes cell division in the root but not in the shoot and the gene that codes for a gamma-glutamylcysteine synthetase involved in glutathiol synthesis, was found to affect root development only in the post-embryonic period (Cheng *et al.,* 1995; Vernoux *et al.,* 2000b).

Unfortunately, very few genes isolated up to now have been observed to be specifically involved in the determination of the primary root meristem as was the *STM* gene for the SAM. One gene, *RCH1*, which encodes a putative receptor kinase as the *CLV1* gene in the SAM, have been shown to be RAM specific (Casamitjana-Martinez *et al.,* 2003). The expression of other *CLAVATA*-like (*CLE*) genes, *CLE19* and *CLE40* was also detected in the RAM (Casamitjana-Martinez *et al.,* 2003; Hobe *et al.,* 2003). A *WUS*-related homeobox gene, *WOX5*, has been found to be expressed in the quiescent center of *Arabidopsis* root (Haecker *et al.,* 2004) as does its rice homolog *QHB* (Kamiya *et al.,* 2003). These findings suggest that a WUS-CLV pathway might exist in the RAM (Fig. 8.12) as in the SAM but the role of the genes must be investigated further (Birnbaum and Benfey, 2004).

Monopteros mutants lack root and hypocotyl in addition to their vascularization defects in the postembryonic phase; during embryogenesis, the first defect is observed at the octant stage with aberrant axialization of the lower tier of the embryo and aberrant hypophysis (Berleth and Jürgens, 1993). Interestingly, the MP protein is very similar to the AUXIN RESPONSE FACTOR 1 (ARF1), a transcription factor that binds auxin-responsive promoter elements, and is thought to mediate responses to auxin (Hardtke and Berleth, 1998). The use of ARF-binding elements (*DR5*) fused to reporter gene (*GUS*) to visualize free auxin, has revealed a maximum in the distal region of RAM and asymmetric auxin distribution was found to establish an organized pattern and polarity in the root meristem (Sabatini *et al.,* 1999). Control of root growth and patterning would be exerted by the PIN auxin efflux protein (Blilou *et al.,* 2005).

5.2.2. Control of radial patterning

Concerning the radial size of the RAM, a range of diverse root morphologies has been described in transgenic root clones expressing the *rol* genes from the root-inducing plasmid of *Agrobacterium* rhizogenes and/or bacterial genes involved in auxin biosynthesis such as *tms1/tms2* from *A. tumefaciens* or their *A. rhizogenes* counterparts. Thick and ramified roots characterized the clones expressing genes for auxin synthesis and provided with high endogenous auxin levels, while roots expressing only the *rol* genes were thin and sparsely ramified (Prinsen *et al.,* 1992; Chriqui *et al.,* 1996; Guivarc'h *et al.,* 1999).

These data pointed to the role of auxin in determining the diameter of the RAM and the subsequent root. The height of the RAM appears also to be regulated by hormones, as reduced levels of cytokinins induced by the overexpression of cytokinin oxidase genes lead to a reduced length of the cell division zone (Werner *et al.,* 2003).

A range of root morphology mutants have also been reported that were affected either in their radial growth patterns and epidermal differentiation (Baskin *et al.,* 1992) or other events (Scheres *et al.,* 1996; Schiefelbein *et al.,* 1997). In the inner part of the root, SHORT-ROOT (SHR), a member of the GRAS family of putative transcription factors, moves outward from the stele and specifies endodermal cell fate. This factor also induces SCARECROW (SCR) which mediates the division of the cortex/endodermal initial daughter cells (Di Laurenzio *et al.,* 1996; Helariutta *et al.,* 2000; Nakajima *et al.,* 2001). In addition to the *MONOPTEROS* (*MP*) gene which is involved in root vascularization (Dolan *et al.,* 1993), a number of *Arabidopsis* homeobox genes (*Ath6*, *8*, *9*, *12*) whose expression patterns indicate that they are important for the differentiation of root vascular tissues. have also been identified (Topping *et al.,* 1991). In the external part of the root, the epidermal cell fate that gives rise to hair and non-hair cells is now well known. At the position where non-hair cells normally form, high levels of *WEREWOLF* (*WER*) promote the expression of both *GLABRA 2*(*GL2*), which inhibits hair cell formation, and *CAPRICE* (*CPC*) which moves laterally to adjacent cells (Masucci *et al.,* 1996; Wada *et al.,* 2002). In the adjacent hair-cell position, high CPC level represses both *WER* and *GL2*, allowing the cells to differentiate into hairs. This negative feed-back involved an intercellular transmission of the inhibitory signal. Later, CTR1, a Raf protein kinase homolog, acts as a negative regulator of hair cell development (Duckett *et al.,* 1994). Little is known about the role of hormones on all these regulatory processes, but global gene analysis has begun to provide a comprehensive inventory of genes which are expressed in roots (Birnbaum *et al.,* 2003); this database will be useful in further understanding the mechanisms of root differentiation.

5.3. LATERAL AND ADVENTITIOUS ROOT FORMATION

5.3.1. Histological Events and the Influence of Auxins

Lateral and adventitious rhizogenesis are *de novo* processes that occur postembryonically from roots or stems. Understanding these issues would make possible a better manipulation of root initiation and architecture. Auxin has, for a long time, been considered as a key root-inducing factor (Haissig and Davis, 1994; De Klerk *et al.,* 1999). It should clearly be interrelated with genes involved in root formation but the connections are not yet clear. It must also be pointed out that the auxin requirement for inducing lateral roots from non-excised roots is higher than from excised roots (Vuylsteker *et al.,* 1998), and that the juvenile or mature state of the plant is critical, as it can condition differential organogenetic responses, in woody species (Hackett, 1987). In addition, nitrate and inorganic phosphate nutrition plays a major role in controlling the development of the root system. Embryonic root and lateral root formation occur *via* some similar mechanisms and share common factors, but lateral root formation is distinct, as shown by mutant analysis. Both *monopteros* and *bodenlos* mutants form normal lateral roots while lacking an embryonic root, and a number of other genes have been identified that affect specifically lateral root development, although most of them implicate auxin as having a crucial role.

Auxin seems to be required for the early steps of cell dedifferentiation and root initiation, then becomes no longer required and to have inhibitory effects on root growth (De Klerk *et al.,* 1999). Lateral root primordia commonly originate endogenously some distance behind the main root apices from dedifferentiation of the parent root pericycle (Fig. 8.11); the endodermis is sometimes also involved. The site at which primordia are initiated is related to the pattern of the vascular system either opposite or adjacent to the protoxylem poles and periclinal division is one of the most common criteria used to define the onset of lateral formation (Esau, 1965; McCully, 1975; Peterson and Peterson, 1986). More exogenous origin can also be observed from the phellogen in tuberized roots. Vascular strands are probably important in determining the sites of primordium initiation through the allocation of trophic (sugars, vitamins), hormonal (auxin, cytokinins) and possibly other signaling factors necessary for primordium initiation (Barlow, 1997). The primordia themselves also interact to determine their relative spacing and a pre-existing primordium actively prevents initiation of a new primordium up to a certain distance along a same rank, perhaps by acting as a sink for nutrients (Charlton, 1982). Lateral root development is similarly submitted to correlative effects and is controlled by the primary root apex that exerts an api-

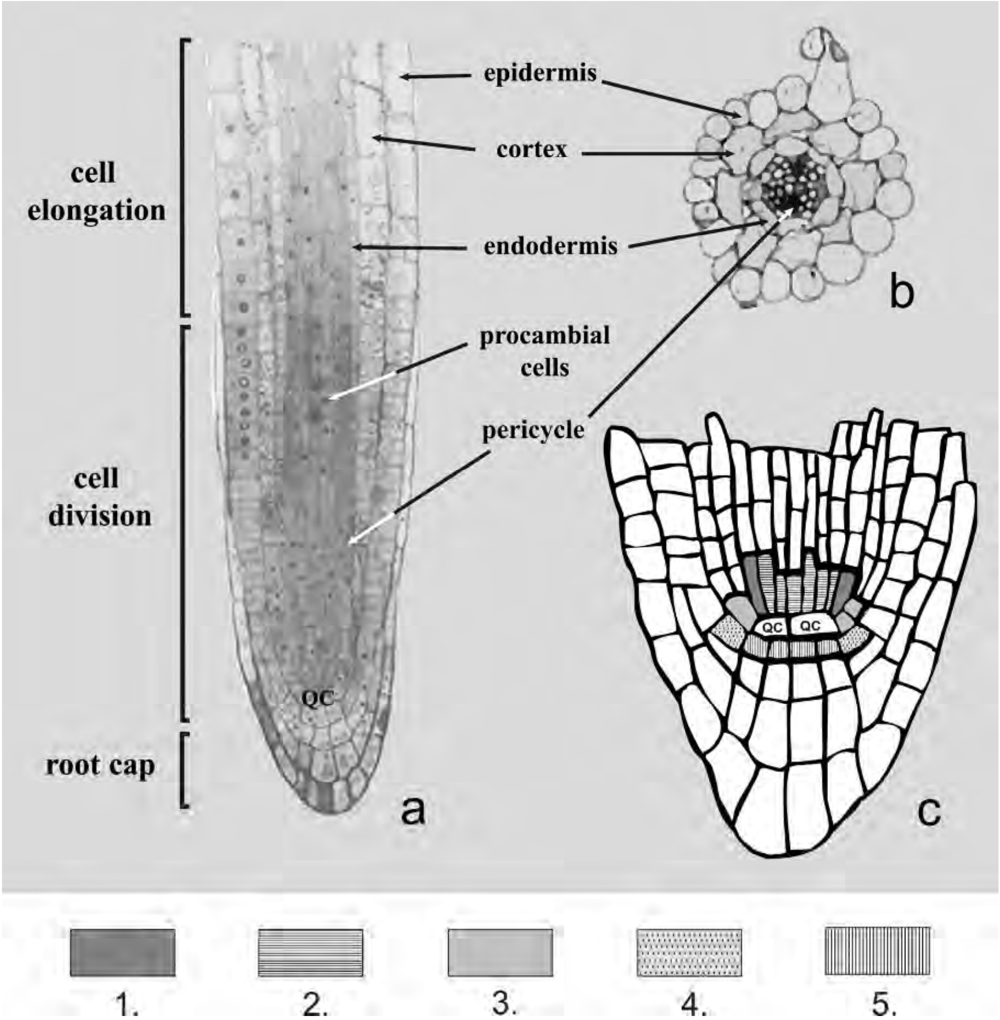

Fig. 8.11 – Structural organization of a root tip.
a – Longitudinal section of an *Arabidopsi*s root tip: cell files defining the different cell types converge onto central cells that constitute the quiescent centre (QC). Cell division occurs above and below the QC, giving rise to cells that elongate to form the root proper, and to the root cap cells, respectively (Courtesy of D. Driss, University P. M. Curie). b – Transverse section in the elongation zone showing the radial organization of the root (Courtesy of R. Atta, University P. M. Curie).
c – Organization of cell types in the root apical meristem: the QC cells are flanked by initials of the various root tissues including columellar initials (5) that permanently renew the root cap, epidermis and lateral root cap initials (4), cortex and endodermis initials (3), pericycle initials (1) and central stele initials (2) (drawn by E. Dubuisson, University P. M. Curie).

cal dominance, probably via cytokinins (Forsyth and Van Staden, 1994) and auxin transport (Muday and Haworth, 1994).

Functional distinction between pericycle cells in the xylem radius file (prerhizogenetic cells or root founder cells) and phloem radius files (non prerhizogenetic cells) is correlated with anatomical differences (Laskowski *et al.,* 1995). The initial stage of lateral root formation begins with a transverse division of pericycle cells adjacent to a xylem pole, resulting in shorter cells that undergo radial expansion before a periclinal division gives rise to two cell layers. Further periclinal divisions of the outer tier of the inner cells increase the number of layers to 2-4 at which stage the lateral root primordium is observed as a bulge of cells. Following emergence through the endodermis and the cortex, cell expansion continues at the base of the

lateral root and the increase of mitotic activity at the lateral root apex indicates the presence of a functional meristem (Laskowski *et al.,* 1995; Malamy and Benfey, 1997). A transgenic *Arabidopsis* line bearing a *CYCLIN B1; 1* promoter fusion with the *GUS* reporter gene, confirmed the unique capacity of pericycle cells adjacent to the protxylem to re-enter the cell cycle then to give rise to root primordia (Himanen *et al.,* 2002). The pericycle was also shown to retain the expression of *CDKA* genes, demonstrating that extensive dedifferentiation is apparently not required for the formation of lateral roots (Hemerly *et al.,* 1993 ; Martinez *et al.,* 1992). In this way, the pericycle has been proposed to be a mono-layered extended meristem (Casimiro *et al.,* 2003).

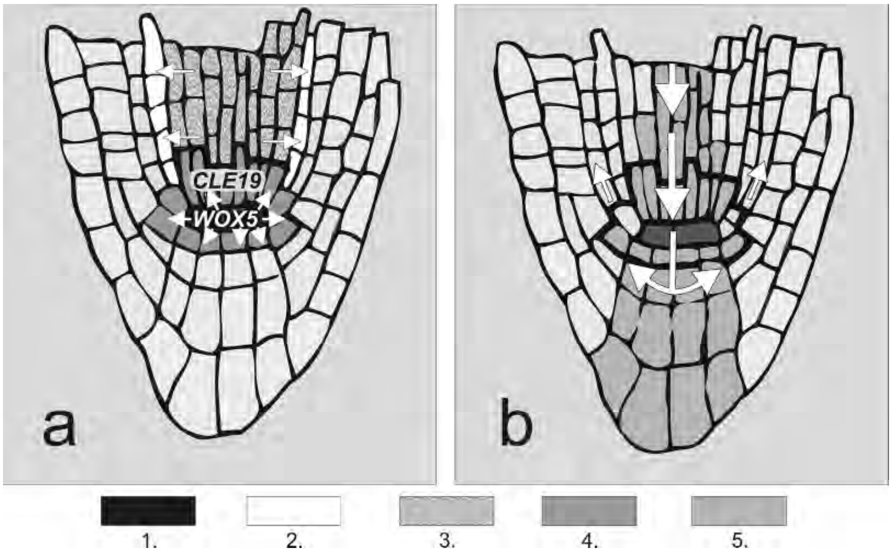

Fig. 8.12 – Intercellular signals that control the position and the size of the stem cell population (initials) in the root apical meristem.
a – *SCR* (2) is expressed in the quiescent centre (1), adjacent ground tissues initials and the endodermis(E). *SHR* (3) is expressed in the stele and is trafficked into the endodermis where it directs cell fate (arrows). QC cells express the WUS–like *WOX5* gene that maintains the surrounding stem cells (4) expressing *CLV3*–like genes (*CLE19*, *CLE40*) in an undifferentiated state. It remains to be determined whether the *CLV*3 and *WUS* homologues in the RAM are connected by a regulatory loop similar to that seen in the SAM.
b – Auxin transport (arrows) and distribution (5) in a root tip: auxin is provided by PIN – dependent auxin transport in the central cells towards the tip, where it accumulates, particularly in the QC and in the root cap. From here, part of the auxin is redistributed by a PIN 2 – dependent auxin route through the outer layers. (drawn by E. Dubuisson, University P. M. Curie).

The histological origins of adventitious root meristems are more diverse. They can be initiated, mainly endogenously, from the phloem parenchyma, from the vascular cambium, from the recent derivatives of the vascular cambium (Barlow, 1986; Lovell and White, 1986) or from the perifascicular cell layer of aerial organs (Chriqui and Bercetche, 1986). The exogenous application of one of the three auxins 2,4-D, NAA and IAA to excised chicory roots induced the formation of lateral root meristems (Vuylsteker *et al.,* 1998). Each of the three auxins induced the formation of new meristems but the subsequent elongation of the neoformed roots was more inhibited by 2,4-D than by NAA and IAA. Higher auxin concentrations seem to be required for adventitious rooting. Similar observations were made from excised *Arabidopsis* root and hypocotyls explants in tissue culture (Atta *et al.,* in preparation)

5.3.2. Genes involved in lateral and adventitious rooting

Mutants that have alterations in endogenous auxin levels, in the auxin transport, or in the auxin-signaling pathway, are also modified in their root forming capacity. The *superroot* (*sur 1* and *sur 2*) mutants of *Arabidopsis* over-proliferate lateral and adventitious root primordia, and contain elevated levels of free and conjugated IAA (Boerjan *et al.,* 1995, Delarue *et al.,* 1998), as do the *rooty* (King *et al.,* 1995), *hls3* (Lehman *et al.,* 1996) and *alf1* (Celenza *et al.,* 1995) mutants that have been isolated independently. The *SUR2* gene encodes a cytochrome that is a modulator of auxin homeostasis

(Barlier *et al.,* 2000). In contrast, the *aberrant lateral root formation alf4 Arabidopsis* mutant lacks lateral and adventitious root primordia (Celenza *et al.,* 1995) and the *adventitious rooting-incompetent* (*rac*) mutant of tobacco has phloem parenchyma unable to organize root primordia in response to exogenous auxin (Lund *et al.,* 1996). The *rac* mutants are blocked in their capacity to activate the *HRGP nt3* promoter which is normally activated by auxin during early root initiation (Lund *et al.,* 1997). The *HRGP nt3* gene encodes a hydroxyprolin-rich glycoprotein expressed in the initial step of lateral and adventitious root formation (Keller and Lamb, 1989). The induction of this gene is one of the initial steps in the sequential program of root initiation as it precedes completion of the first cell division during primordium formation (Vera *et al.,* 1994). When *AUX1*, a gene responsible for auxin influx, is mutated, significant inhibition of lateral rooting is observed. Genes from the *PIN* and *PID* families responsible for auxin efflux are also required for lateral root formation. Polar auxin transport from the shoot to the root tip is important for lateral root development at the whole plant level (Casimiro *et al.,* 2001, Bhalerao *et al.,* 2002). Genes from the *AUX/IAA* family, which encode proteins involved in auxin-responsive gene expression, are also required as shown by the strong expression of the *DR5* element at pericycle sites involved in root formation; the high *GUS* expression driven by *DR5* indicates auxin accumulation at the root forming sites (Himanen *et al.,* 2002). Precise analyses of cell cycle events associated with *Arabidopsis* lateral root initiation have revealed a key role for the *KRP2* gene, one of the recently identified CDK inhibitors of entry into the S phase of the cell cycle. *KRP2* is highly expressed under root treatment with the inhibitor of auxin transport NPA and its expression dramatically declined upon auxin treatment. *In situ* hybridization shows that *KRP2* transcripts accumulated in pericycle cells not involved in root formation and overexpression of *KRP2* reduces lateral rooting. Consequently, auxin levels in the main root apex might control lateral root initiation *via* the transcriptional regulation of *KRP2* (Himanen *et al.,* 2002 ; Casimiro *et al.,* 2003).

Some other genes with functions more or less identified, are also known to be involved in lateral and/or adventitious root formation. Among them, *LRP1* (*LATERAL ROOT PRIMORDIUM 1*) from *Arabidopsis* is activated only during lateral and adventitious root primordium formation and is turned off prior to emergence (Smith and Federoff, 1995). The *RS1* gene of tomato was activated in very early initials of lateral and adventitious roots, and persisted until shortly after the emergence from the parent tissue (Taylor and Scheuring, 1994). The *rtcs* maize mutants completely lack the formation of crown and lateral seminal roots (Hetz *et al.,* 1996). The *octopus* (*oct*) mutant of *Arabidopsis* initiates lateral root primordia but cannot maintain meristematic activity, the activity of the primary root meristem being unaffected. The *OCT* gene has been tagged by a *GUS*-promoter trap construct and the *GUS* expression indicated that the *OCT* gene is expressed in the root vascular system (Ferreira *et al.,* 1994).

Interestingly, some cell wall signals that stimulate root formation are also present during early development of somatic embryos (Pennell *et al.,* 1995), suggesting common regulatory pathways in both morphogenetic events. However, little information is available on this topic. The listing of genes potentially involved in lateral root formation is in progress (Casimiro *et al.,* 2003) but a full understanding of this morphogenetic event has not yet been achieved. Rapid advances are expected in the coming decade with the use of combined genetic-cell-biological approaches.

5.4. THE QUESTION OF ROOT AND SHOOT DETERMINATION

For some species, development of buds on roots (Peterson, 1975) makes possible natural vegetative propagation by root cuttings. Those buds frequently arise endogenously like lateral roots. They may originate from the pericycle in young roots, with strong similarities with root primorda (Bakshi and Coupland, 1959, Projetti and Chriqui, 1986a). From older roots, they may be initiated exogenously from callus arising from the phellogen (Murray, 1957) or directly from the phellogen (Projetti and Chriqui, 1986a). They often arise at the vicinity of lateral roots and may become connected to the lateral root trace. Conversely, development of adventitious roots on various aerial organs is also possible (Barlow, 1986). The occurrence of such adventitious shoots and roots indicates that some mature stem and root cells are not irreversibly determined. When the *WUS* gene is over-expressed in roots using a Cre-*lox* based expression system, the root tips formed ectopic shoots or auxin-dependent somatic embryo-like structures; this suggests that RAM cells can be redirected to at least two different developmental pathways (Gallois *et al.,* 2004). Only a limited

number of experimental cases have been reported in which the same cell type was able to organize directly either a root or a shoot apical meristem in response to hormones added exogenously. This was the case for the root pericycle cells and for the leaf perifascicular cells of *Rorippa sylvestris* (Projetti and Chriqui, 1986a, b). Evidence for a common developmentally labile early stage from which shoot or root meristems would become determined is still lacking, but it could be expected that combination of *in vitro* cultures and molecular approaches, with the new tools offered by genes involved in shoot or root apical meristem identity, will allow advances in this field. The role of hormones in determining meristem identity is not yet understood, but data on cytokinin-deficient plants over-expressing cytokinin oxidases have indicated opposite functions of cytokinins in the regulation of SAM and RAM activity (Werner *et al.,* 2003).

6. SECONDARY MERISTEMS AND RADIAL GROWTH

In most dicotyledons and in gymmosperms, expansion growth of stems and roots occurs through the establishment and the functionning of 2 to 4 internal meristematic layers dividing periclinally. These are called secondary meristems or cambium. Cambia were formerly divided into vascular cambium giving rise to secondary xylem and phloem, and cork cambium that produces cork. The cork cambium is now usually named phellogen, and the term cambium is preferably used for vascular cambium.

6.1. ORIGIN AND FUNCTION OF THE VASCULAR CAMBIUM

The vascular cambium allows the development of tree-like forms and ensures the perennial life of trees through the regular renewal of secondary xylem and phloem. It is also present in many herbaceous dicotyledons. The origin of cambial layers is different in stems and in roots. At early stages of leaf initiation from the SAM, some L3 cells in the leaf founder regions establish a procambium (provascular strand) that will produce the primary xylem and phloem cells of the leaf, and consequently, of the stem. Part of the procambial cells subsist, recover secondarily a mitotic activity and divide periclinally, giving rise to the intrafascicular cambium. The signal for cell division spreads to the neighbouring parenchyma cells, changing them into interfascicular cambium, so that the cambium forms a complete ring of dividing cell layers and gives rise to cylinders of secondary tissues. In roots, strands of primary xylem and phloem are alternate, and the vascular cambium is initiated by divisions of the procambial cells that remained undifferentiated between the primary xylem and the primary phloem. Thus, at the beginning, the cambium has the form of strips whose number depends on the type of root organization. Subsequently, the pericycle cells located behind the xylem poles reactivate as a cambium, and then the cambium completely encircles the primary xylem core. As for the shoot cambium, the root cambium produces secondary phloem and xylem by periclinal divisions and increases in circumference by anticlinal divisions (Esau, 1965). Mature vascular cambium is usually constituted of two distinct cell types: the ray initials and the elongated fusiform initials, both contributing to the whole cambial fabric and to the horizontal and vertical systems of the secondary vascular tissues (Catesson, 1994).

In many trees, the cambium presents alternate periods of meristematic activity and arrested growth, the timing of which being under environmental and genetic control and giving rise to successive rings on the wood. The annual cycle of cambial activity is accompanied by an arrest of cells in the G1 phase of the cell cycle during winter arrest (Mellerowicz *et al.,* 1989).

Auxin is considered as the main phytohormone involved in the regulation of cambial activity. It has a stimulatory effect on cambial cell division (Zakrzewski *et al.,* 1983, Sundberg *et al.,* 1993). It also favours the formation of fusiform initials rather than radial initials (Lev-Yadun, 1995). Auxin concentration gradients, involving polar auxin transport through the *AUX1*-like family of influx and *PIN1*-like family of efflux carriers, regulates seasonal activity of the cambium as well as xylem maturation. The different fates of inside (xylem) and outside (phloem) cambial derivatives might originate from unequal auxin distribution (Schrader *et al.,* 2003). In addition, auxin is crucial in inducing procambial cell formation as demonstrated by the analysis of *pin 1* and *gnom* mutants (Gälweiler *et al.,* 1998; Koizumi *et al.,* 2000). Cytokinin is also essential for promoting the division of procambial cells as revealed by the *wol/cre1* mutants in which all procambial cells differentiate into protoxylem, the mutated gene encodes a cytokinin receptor (Mähonen *et al.,* 2000; Scheres *et al.,* 1995). However, little is known at the molecular level about how auxin and cytokinins induce procambium, then cambium formation.

Recently, a regulation by miRNA of the *PHAVOLUTA* gene, a gene involved in leaf adaxial identity, has been shown to be involved in vascular cambium cell determination (McHale and Koning, 2004). A number of other genes such as *ATHB8* (Baima *et al.,* 1995) are expressed in procambial cells but their precise role in procambium and cambium formation is not yet understood.

Active cambial cells *in planta* are provided by both auxin (Schrader *et al.,* 2003) and cytokinins (Boucheron *et al.,* 2001); they have been shown to express some of the cell cycle genes that are also expressed in the primary apical meristems (Boucheron *et al.,* 2001). This inherent cell cycling capacity might explain the particular ability of cambium to form rapidly a callus both following wounding and in tissue culture without dedifferentiation processes. It could also explain the particular ability of cambial cells to be transformed by *Agrobacterium* strains (Chriqui *et al.,* 1988; Guivarc'h *et al.,* 1993).

6.2. PHELLOGEN ORIGINS AND PERIDERM FORMATION

In old stems of herbaceous dicotyledons, as in woody dicotyledons and conifers, the phellogen arises beneath the epidermis, in a subepidermal or in a deeper position, from cortical cells that re-enter a mitotic activity and divide periclinally (Esau, 1965). The phellogen produces externally suberified cells (phellem), and internally, phelloderm cells. These tissues form the periderm which is either persistent, or is regularly sloughed off and renewed (the outer bark or rhytidome). In roots, phellogen has a deeper origin and arises usually from the pericycle cells that undergo periclinal and anticlinal divisions. Periclinal divisions cause an increase of the radial extent of pericycle layers. The combined increase in thickness of the vascular tissues and of the pericycle derivatives forces the cortex outward. The cortex does not undergo an increase in circumference but becomes ruptured and sloughed off together with the epidermis and endodermis. The phellogen layers stay in the outer part of the pericycle derivatives and produce phellem toward the outside and phelloderm toward the inside (Esau, 1940). Nothing is known concerning the genetic control of the differentiation of these tissues.

6.3. OTHER TYPES OF RADIAL GROWTH

Some plants, like monocots, lack secondary meristems but have intercalary meristems. These meristems are formed of actively dividing cells that differ from apical meristems and are located between more and less differentiated tissues at the base of each internode. Such intercalary meristems do not produce vascular tissues but participate in the radial growth of the stem. They are the result of a secondary meristematic activity that is not present in the embryo and originates from a process of natural dedifferentiation.

7. THE CELL CYCLE

7.1. CELL PROLIFERATION, POLYPLOIDY AND DEVELOPMENT

The various morphogenetic processes in plant development involve cell division activity, both in *planta* and in tissue culture. Cycling cells are found mainly in the primary shoot and root meristems, secondary meristems (vascular cambium and phellogen), and in specific cell types such as stomata, oil glands or glandular trichomes. Cells are also induced to divide at the onset of any type of adventitious organ formation and cell cycle reactivation is a prerequisite for callusing and regenerating processes in tissue culture. The eukaryotic cell division cycle is usually divided into four distinctive phases (Howard and Pelc, 1953): G1 (G for gap, time interval) during which the cell grows with a 2C DNA content, S (synthesis phase) during which the nuclear genetic information is replicated, G2 (4C DNA content), when further growth in preparation for division occurs, and M (mitosis), in which the cellular contents are partitioned between the daughter cells. The S and M phases require more energy for their completion than the G1 and G2 phases. Sucrose added to excised pea root tips rapidly stimulates the entry of G1 cells into S, and of G2 into M (Van't Hof, 1973). This observation is probably important for understanding the effect of sucrose added in tissue culture medium to initiate cell dedifferentiation. It also points out that major controls operate at the transition points from G1 to S and from G2 to M.

The duration of S and M phases is rather constant for a given species under a standard set of conditions. Both the shoot and root meristems are mosaics of fast and slow cycling cells. Modulations of the total duration of the cell cycle are mainly due to variations in the duration of the G1 phase. The G1 phase in active shoot apical meristems is always longer than

the S + G2 + M phases (Nougarède and Rembur, 1985). Within meristems, once S phase is initiated, genes for the completion of mitosis are activated and cell division automatically follows.

In the vegetative shoot apical meristem of various species, quantitative data about the cell-doubling times in the central (CZ) and peripheral (PZ) zones indicate that the cell cycle duration is always shorter in the PF. Changes in the cell cycle are characteristic of developmental switches. For example, in *Chrysanthemum segetum*, the mean cell doubling time is 139 h in the CZ and 48 h in the PZ; at the switch to reproductive phase, the activation of the CZ is marked by a decrease of the cell cycle duration to 54 h and a loss of the cytophysiological zonation of the SAM (Nougarède and Rembur, 1977); this is achieved by a shortening of the G1 phase (Nougarède and Rembur, 1978). Cell sizes in the vegetative PZ are also significantly smaller than those in the CZ while they become more uniform across the prefloral shoot apices. It was hypothesized that cell size is a component of developmental programs and that a critical cell size is required to commit the cells to enter the S phase for a new round of division. A tissue-specific size control would operate at both late G1 and late G2 checkpoints (Francis, 1998), but the extent to which signal transduction chains impinge on the plant cell cycle with consequences for cell size is still to be analyzed.

During temporary arrests of meristematic activity such as bud winter dormancy, axillary bud inhibition or seed dormancy, cells were found to stop the cell cycle in a prolonged G_1 (G_0) phase (Rembur, 1972; Brossard, 1973; Nougarède and Rondet, 1975; Cottignies, 1979). At the transition to flowering, the cell cycle shortens (Miller and Lyndon, 1975; Nougarède and Rembur, 1978). It remains unclear whether changes in the cell cycle *in planta* cause developmental switches or whether they are consequences of developmental switching by other mechanisms. The same question arises concerning the various types of regeneration that can be induced in tissue culture that require cell proliferation.

In the non-cycling differentiated parts of plants, supernumerary rounds of DNA synthesis can occur in cells that have lost their mitotic ability (Fig. 8.13). This process of endopolyploidisation (endoreduplication) is rather frequent in higher plants. In such polysomatic species, nuclear DNA contents can reach very high levels, for example up to 32C in W38 tobacco pith (Brossard, 1975) and *Arabidopsis thaliana* leaves (Galbraith *et al.,* 1991), and cases of polyteny have been reported in embryonic tissues (d'Amato, 1952). Endoreduplication also occurs in seed or plant tissues with high metabolic activity, suggesting that it might provide a mechanism whereby cells increase the availability of DNA template and thus increase the level of gene expression (Grafi, 1998). The presence of polyploid cells in initial explants is thought to have consequences for abnormal nuclear behaviour in tissue culture including somaclonal variation, aneuploidy and nuclear fragmentations (Nuti Ronchi *et al.,* 1973; Brossard, 1975; Bercetche *et al.,* 1993). In contrast, the non polysomatic species such as *Populus* spp. (Jehan *et al.,* 1994), *Eucalyptus globulus* (Azmi *et al.,* 1997) or *Crepis capillaris* (Brossard, 1978) are characterized by cell cycle arrest in a 2C level at the onset of cell differentiation. The ploidy levels of the various tissues can vary within a polysomatic species. For example, W38 tobacco mesophyll cells remain usually diploid, while mature pith tissue is a mosaic of ploidy levels from 2C to 32C. The biological significance of endoreduplication is still unknown. It has been hypothesized that it is a means whereby small genomes can correctly support rapid cell growth and that the final size of a cell is linked to its DNA content. In some cases, such as trichomes and hypocotyl cells of *Arabidopsis*, endoreduplication stops very early during cell differentiation when cells elongate (Melaragno *et al.,* 1993) but the existence of non-polysomatic species contradicts the hypothesis. Whatever it may be, the current hypothesis is that endopolyploidization provides a mechanism for increasing cell size and gene transcription (Sugimoto-Shirasu and Roberts, 2003).

7.2. MOLECULAR CONTROL OF THE CELL CYCLE

As in all eukaryotes, progression through the plant cell cycle (Fig. 8.14) is controlled by cyclin-dependent kinases (CDKs) that bind to positive regulators called cyclins and regulate the transition points from G1 to S and G2 to M. Intensive efforts over the past ten years have identified a range of genes encoding CDK-like proteins and cyclins in various plant species and detailed reviews have been published recently (De Veylder *et al.,* 2003; Dewitte and Murray, 2003). Interestingly there is accumulative evidence that many of the controlling factors are highly conserved and similar in mammals and plants, including not only the CDKs and the cyclins, but also retinoblastoma-like proteins,

transcription factors E2F and D-type cyclin kinases (Huntley and Murray, 1999; Shen, 2002).

CDKs are serine/threonine kinases that control progression of the cell cycle in all eukaryotes. Their activity is regulated both by association with cyclin regulatory submits and by phosphorylation/ dephosphorylation events. They are characterized by specific motifs involved in cyclin- and ATP-binding and have been initially separated into two main classes:

- the PSTAIRE class containing the PSTAIRE sequence within a cyclin interaction motif. An example from this group is the CDK A protein from *Arabidopsis*;
- the non-PSTAIRE class having a variant motif (PPTALRE or PPTTLKE) at the equivalent position to that in the *Arabidopsis* CDK B proteins.

In *Arabidopsis*, the *CDC2a* gene (CDK A) was found to be expressed during both in G1-to-S and in G2-to-M transitions, i.e. in a cell-cycle-phase independent pattern, while the *CDC 2b* (CDK B) gene is more associated with the progression through G2 (Magyar *et al.,* 1997; Menges and Murray, 2002) (Fig. 8.7). It has been reported that the *CDC2a* gene is expressed not only in cycling cells but also in cells showing competence for division (Hemerly *et al.,* 1993; Martinez *et al.,* 1992; Boucheron *et al.,* 2001), suggesting that it could be a component of the cell totipotency. In addition, *CDC2a* was found to have some involvement in the orientation of cell division planes and cell size control (Hemerly *et al.,* 1993). More recently, three other CDK classes (C, D and E) have been identified (Joubes *et al.,* 2000) to which has been added the distantly related CDK activating kinase CAK1, renamed CDK F1 (Vandepoele *et al.,* 2002).

The plant cyclins were initially put into groups reflecting their similarities with the mammalian cyclins A, B, C and D (Mironov *et al.,* 1999), the C-type cyclins being sparsely documented. Recently, the number of cyclins defined in *Arabidopsis* has expanded to 49 distributed in 8 classes and 23 subgroups (Torres Acosta *et al.,* 2004; Wang *et al.,* 2004). The expression of the main cyclin genes has been studied, giving information on how their transcripts accumulate during the various phases of the cell cycle (Menges *et al.,* 2002, 2003, 2005) (Fig. 8.14). The A- and B-type cyclin accumulation is mainly phase dependent. The B-type cyclins are mitotic cyclins correlated with the G2/M transition. Results are less strict for A-type cyclins, a group in which some cyclins are expressed uniformly throughout the cell cycle while others start accumulating at the end of the G1 phase or during the S phase. The majority of D-type cyclins display fairly constant expression levels throughout the cell cycle and, by analogy with their animal homologs, have been proposed to control the G1-to-S transition (Dahl *et al.,* 1995; Soni *et al.,* 1995). The genes encoding CYCD cyclins are induced at a specific time during cell cycle re-entry but generally remain expressed at a constant level in actively dividing cells (Huntley and Murray, 1999). However, the transcripts of two tobacco cyclin genes, *CYCD2* and *CYCD3,* accumulate during mitosis in synchronized BY-2 cells (Sorrell *et al.,* 1999). The apparent discrepancies between results probably reflect the fact that only some of the genes controlling the cell cycle have been identified so far and that much work is still to be done in order to have a fully comprehensive understanding of the phenomenon. In addition, the abundance of cyclin mRNA and protein are sometimes not correlated with the level of the activity (Mironov *et al.,* 1999). Another *CYCD2*-type cyclin from *Arabidopsis* is expressed only during lateral root formation (Mironov *et al.,* 1999). Both CDK A and B can be partners of specific CYCD (Healy *et al.,* 2001; Kono *et al.,* 2003) and D-type cyclins show multiple patterns of expression and regulation, as shown by microarrays studies: during cell cycle re-entry, two distinct expression timing are observed : CYCD5;1 and CYCD3;3 accumulate early in G1v and decline markedly as cells move towards S, whereas CYCD4;1, CYCD4;2 and CYCD3;1 accumulate late in G1 and peak at the G1/S transition (Menges *et al.,* 2005).

The destruction of cyclins at specific points of the cell cycle depends on the presence of either a destruction box motif (CYCA and CYCB) or specific sequences (PEST) which are thought to be signals for rapid proteolysis (CYCD). In addition to cyclins, other proteins have been isolated by their ability to interact with CDKs. In this category, CKS1 from *Arabidopsis* binds to both CDK A and CDK B; it is expressed during the mitotic and presumably also the endoreduplication cycles (De Veylder *et al.,* 1997; Jacqmard *et al.,* 1999). Protein inhibitors of CDKs (KRPs, also known as ICKs for inhibitors of CDK) play also important roles in modulating CDK activity. One of these inhibitors (KRP1/ICK1) interacts with both CDC2a and CYCD3 (Wang *et al.,* 1998). *KRP1* is induced by ABA and leads to a decrease in CDK activity, suggesting that KRP1 may mediate the cytostatic effect of abscisic acid. The distinct KRPs may have differential roles in regulating the mitotic cycle and/or the endomitotic process (Ormenese *et al.,* 2004).

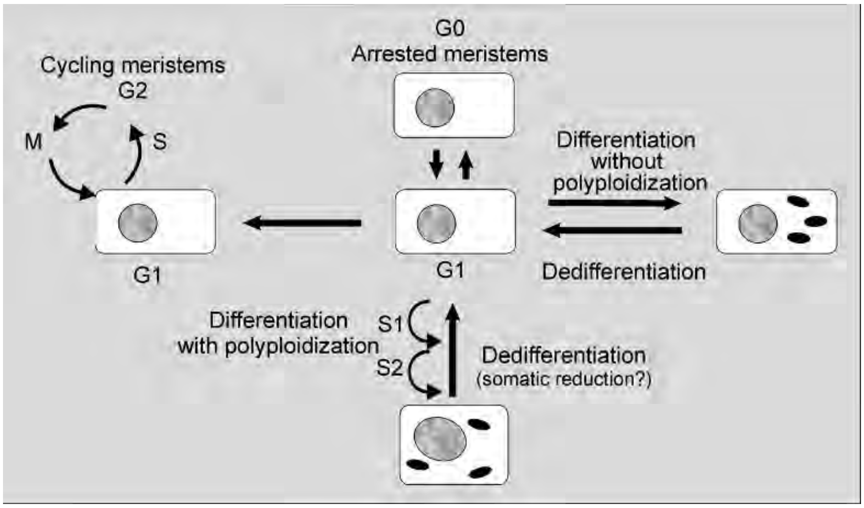

Fig. 8.13 – Options for G1 cells during plant development.
Newborn G1 cells can start another round of division when located within meristems or arrest during periods of dormancy . They can also differentiate either in a G1 state giving rise to non-polyploid tissues or undergo several rounds of DNA synthesis without dividing, giving rise to polyploid cells. The differentiated cells can re-enter the cell cycle given the appropriate signals (redrawn from Den Boer and Murray, 2000b, by E. Dubuisson, University P. M. Curie).

Concerning CDK phosphorylation as a control mechanism in plants, it was found that cytokinin-depleted cells arrested in G2 contained CDK complexes with reduced kinase activity and high phosphotyrosine content. They could be stimulated to re-enter the cell cycle upon addition of cytokinins or by expression of the *CDC25* gene from fission yeast (John, 1998). Resumption of the cell cycle results in tyrosine dephosphorylation and kinase reactivation. The yeast CDC25 phosphatase being highly specific for the Tyr15 of CDKs, the results suggest that the triggering of Tyr15 dephosphorylation is an essential function of cytokinins in the plant cell cycle. The *CDC25* gene has also been expressed in transgenic tobacco plants and root cultures, and in both cases the cells were found to divide at a reduced size (McKibbin *et al.,* 1998), supporting the importance of Tyr15 phosphorylation in the timing of mitosis. Recently, the first CDC25 phosphatase was characterized in plants (Landrieu *et al.,* 2004a and b). Growing evidence suggests that pRB-like proteins are also among the important factors in the plant cell cycle. The pRB (retinoblastoma protein) is central to the G1-to-S transition in mammals. Phosphorylation of pRB by cyclin D-dependent kinases renders it inactive as a repressor of the S phase but promotes DNA replication. pRB proteins have been also found in plants (Huntley *et al.,* 1998; Vandepoele *et al.,* 2002). The observation that a pRB protein in maize leaves is highly produced in differentiating but not in proliferating cells suggests that it could be involved in the suppression of cell division during differentiation (Huntley *et al.,* 1998). Other cell cycle partners are E2Fa, an activator of gene expression stimulating cell division (De Veylder *et al.,* 2002) and controlling CDK B expression (Boudolf *et al.,* 2004), as well as the WEE 1 kinase which is a candidate to inhibit M-phase specific CDK activity during endoreplication (Sun *et al.,* 1999). At least one inhibitor (KRP1) can block entry into mitosis but allow S-phase progression, causing endoreplication (Weinl *et al.,* 2005). Among genes encoding conserved and plant-specific cell cycle regulators identified so far in *Arabidopsis*, there are 29 CDKs, 49 cyclins, 7 KRPs (inhibitors of the CDK-cyclins complex) and 2 CDK subunits (Menges *et al.,* 2005). The considerable advances made in recent years in understanding the basic mechanism that regulate the cell cycle have made it possible to propose a model (Fig. 8.14) that integrates current knowledge. However, a number of important questions have still to be solved to understand how cell division is initiated, how endoreduplication is regulated and how cells exit the cell cycle to differentiate. The importance of all the cell cycle

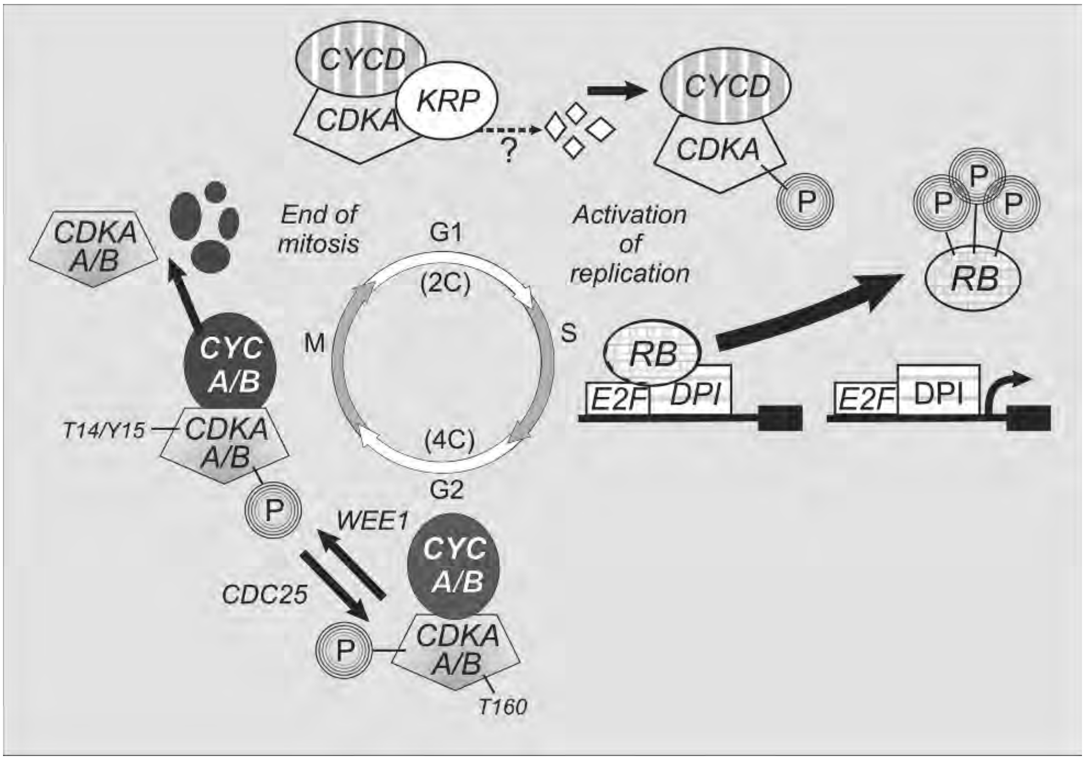

Fig. 8.14 – Key events in cell cycle progression.
During the G1 phase, the activity of CDKs is modulated by their association with CYC D2 and CYC D3, *CYCD*2 and *CYCD*3 genes responding to sucrose and *CYCD3* being also induced by cytokinins. CDK inhibitors (KRP) prevent kinase activity of cyclin – CDK complexes and one of them is induced by abcisic acid. In late G1, rising levels of CYCD-CDKA kinase activity lead to phosphorylation of retinoblastoma (RB). Phosphorylation renders RB inactive, the E2F transcription factor is no longer repressed and S–phase genes are transcribed. Cells that approach M phase contain inactive CDK–CYCA/B complexes, required removal of inhibitory phosphate(P) on T14 and Y15 by a CDC25 phosphatase. The active complex triggers entry into prophase and the A/B cyclins are degraded at the end of mitosis. (redrawn by E. Dubuisson, University P.M. Curie).

parameters identified so far in plant development has only recently begun to receive attention. Manipulation of cell cycle genes to modify plant growth and development has started recently (Doerner *et al.,* 1996; den Boer and Murray, 2000b; Wyrzykowska *et al.,* 2002) but how the cell cycle regulators are linked to genes that co-ordinate meristem function is not yet understood. A change in shoot apical meristem structuration has recently been found in transgenic tobacco lines overexpressing the *CYCD3* genes, suggesting that at least this gene interacts with genes involved in the identity of the central zone of the SAM (Boucheron *et al.,* 2005). Concerning tissue culture, the presence of pre-existing cycling cells in the explants has considerable consequences for the ability of both dedifferentiation (Boucheron *et al.,* 2001) and integration of foreign DNA (An *et al.,* 1985; Guivarc'h *et al.,* 1993).

Visualizing cell cycle activity can be helpful for several aspects of tissue culture. In such cases, *in situ* hybridization or the use of transgenic lines harbouring promoter: *GUS* gene fusion can be done with some of the main cell cycle genes. The well-characterized marker is CYCB, whose promoter activity correlates well with the corresponding mRNA localization and marks cell cycle progression through late G2 to M phase. Histone H4, E2FA and CYC D3 are good candidates to mark the G1 to S transition. In addition, while CDKA promoter activity reflects a state of competence for cell division, CDKB is identified as a G2 to M phase-dependent kinase.

7.3. HORMONAL CONTROL OF THE CELL CYCLE

The growth regulators auxin and cytokinin are regarded as essential for cell division although their precise role in this process is not yet fully understood (den Boer and Murray, 2000a). Evidence for their involvement derives largely from work with callus and suspension cultures and the extrapolation of the conclusions from these systems to organized meristems is little documented. Auxin has for a long time been considered to be involved in the initiation of DNA synthesis and in cell enlargement, allowing

the cells to reach the critical size required to initiate a new cycle. Cytokinins were mostly considered to be involved in the process of mitosis and a combination of auxin and cytokinin is frequently required to reinitiate cell proliferation in tissue culture (Skoog and Miller, 1957). Auxin alone increases the level of a CDK protein in cultured tobacco cells but a cytokinin is required for activation of this kinase (John *et al.,* 1993). The *CDC2A* promoter is inducible by auxins and, to a lesser degree, by cytokinins (Hemerly *et al.,* 1993). Involvement of cytokinins at the G2-to-M transition has been demonstrated by analyses of endogenous hormones during the cell cycle of synchronized tobacco BY-2 cells. Peaks of zeatin and dihydrozeatin were observed at the end of the S phase and during mitosis; the cell cycle blocked in G2 by lovastatin, a cytokinin biosynthesis inhibitor, can be reversed by addition of exogenous zeatin (Laureys *et al.,* 1998). It has been suggested that cytokinin-depleted cells accumulate CDK-cyclin complexes that are inactive due to phosphorylation of Thr 14 and/or Tyr 15 regulatory residues of the CDK, addition of cytokinins of tyr dephosphorylation being able to restore the kinase activity (Zhang *et al.,* 1996). Cytokinins seem also to regulate the G1-to-S transition. In *Arabidopsis* cell cultures, it has been found that *CYCD3* is induced at the end of the G1 phase by cytokinins and sucrose (Riou-Khamlichi *et al.,* 1999 and 2000). The cytokinin overproducing *amp1* mutant has *CYCD3* mRNA levels that are enhanced two- to three-fold, further supporting the evidence for a link between cytokinins and *CYCD3* expression. Because cytokinins are also involved in the expression of the *KNAT1* and *STM* genes encoding homeodomain proteins that prevent differentiation and activate cell division (Frugis *et al.,* 1999 ; Rupp *et al.,* 1999), it could be expected that links exist between cytokinins, cell cycle genes and developmental genes, but the Other growth regulators, such as gibberellic acid and abscisic acid, may also influence the mitotic cycle. For example, CDKs accumulate in response to gibberellins in specific tissues (Sauter, 1997) and *KRP1* expression is induced by ABA (Wang *et al.,* 1998). However it is not clear whether these hormones are of primary importance in maintaining or blocking the cycle or whether they only modify cycles already in progress. In NPA-synchronized *Arabidopsis* roots submitted to auxin treatment to study the kinetics of cell cycle activation at early stages of lateral root initiation, it was found that the *KRP2* gene was down-regulated prior the onset of *CYCB* expression; *in situ* hybridization revealed that only the pericycle cells located behind the xylem poles were able to express *CYCB* together with auxin accumulation while the phloem pericycle cells were expressing *KRP2* and were unable to reactivate (Himanen *et al.,* 2002).

8. CONCLUSION

The value of *Arabidopsis* as a model plant together with the systematic genomic approaches now combined with functional genomic analysis have begun to shed light on the massive changes in gene expression that occur at each developmental stage. Recent molecular genetic studies have shown that many results with *Arabidopsis* are largely applicable to other eudicots or grass plants. Furthermore, a number of developmental genes, including various genetic (transcription factors) and epigenetic (micro RNAs, RNAi, chromatin remodeling factors) regulators have already been identified. In the future, the careful combination of new technologies will produce high-resolution information allowing us to start probing the complexity of the gene regulation network in plant development. There is no doubt that such findings will also be helpful in understanding the behaviour of excised tissues or organs placed in tissue culture and the role of culture medium components in controlling developmental genes.

REFERENCES

ABE M., TAKAHASHI T. & KOMEDA Y. 1999 Identification of a cis-regulatory element for L1 layer-specific gene expression, which is targeted by an L1-specific homeodomain protein. Plant J. 26, 487-494.

AIDA H., ISHIDA T., FUKASI H., FUJISAWA H. & TASAKA M. 1997 Genes involved in organ separation in *Arabidopsis*: an analysis of the cup-shaped cotyledon mutant. Plant Cell 9, 841-857.

AIDA H., ISHIDA T. & TASAKA M. 1999 Shoot apical meristem and cotyledon formation during *Arabidopsis* embryogenesis: interaction among the CUP-SHAPED COTYLEDON and SHOOT MERISTEMLESS genes. Development 126, 1563-1570.

ALVAREZ J., GULI C.L., YU X. & SMYTH D.R. 1992 Terminal flower: a gene affecting inflorescence development in *Arabidopsis thaliana*. Plant J. 2, 103-116.

AN G. 1985 High efficiency transformation of cultured tobacco cells. Plant Physiol. 79, 568-570.

ARAKI T. 2001 Transition from vegetative to reproductive phase. Curr. Opin. Plant Biol. 4, 63-68.

ARAKI T. & KOMEDA Y. 1993 Analysis of the role of the late-flowering locus, GI, in the flowering of *Arabidopsis thaliana*. Plant J. 3, 231-239.

AUKERMAN M.J. & SAKAI H. 2003 Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. Plant Cell 15, 2730-2741.

AZMI A., NOIN M., LANDRÉ P., PROUTEAU M., BOUDET A.M. & CHRIQUI D. 1997 High frequency plant regeneration from *Eucalyptus globulus* Labill. hypocotyls: Ontogenesis and ploidy levels of the regenerants. Plant Cell Tissue Organ Cult. 51, 9-16.

BAIMA S., NOBILI F., SESSA G., LUCCHETTI S., RUBERTI I. & MORELLI G. 1995 The expression of *Athb-8* homeobox gene is restricted to provascular cells in *Arabidopsis thaliana*. Development 12, 4171-4182.

BAKSHI T.S. & COUPLAND R.T. 1959 An anatomical study of the subterranean organs of *Euphorbia esula* in relation to its control. Can. J. Bot. 37, 613-620.

BANNO H., IKEDA Y., NIU Q-W. & CHUA N. H. 2001 Overexpression of *Arabidopsis* ESR1 induces initiation of shoot regeneration. Plant Cell 13, 2609-2618.

BARLEY R. & WAITES R. 2002 Plant meristems: the interplay of KNOX and gibberellins. Curr. Biol. 12, R696-R698.

BARLIER I., KOWALCZYK M., MARCHANT A., LJUNG K., BHALERAO R., BENNETT M., SANDBERG G. & BELLINI C. 2000 The SUR2 gene of *Arabidopsis thaliana* encodes the cytochrome P450 CYP 83B1, a modulator of auxin homeostasis. Proc. Natl. Acad. Sci. USA 97, 14819-14824.

BARLOW P. 1986 Adventitious roots of whole plants: their forms, functions and evolution. pp. 68-110 in M.B. Jackson (ed.), *New root formation in plants and cuttings*. Martinus Nijhoff Publ., ISBN 90 247 3260 3.

BARLOW P. 1997 Positional controls in root development. in P.W. Barlow and D.J. Carr (eds). *Positional controls in plant development.* Cambridge University Press, 281-318. ISBN 052125406X.

BARTON M.K. 1998 Cell type specification and self-renewal in the vegetative shoot apical meristem. Curr. Opin. Plant Biol. 1, 37-42.

BARTON M.K. & POETHIG R.S. 1993 Formation of the shoot apical meristem in *Arabidopsis thaliana*: an analysis of development in the wild-type and shoot meristemless mutant. Development 119, 823-831.

BASKIN T.I., BETZNER A.S., HOGGARD R., CORK A. & WILLIAMSON R.E. 1992 Root morphology mutants in *Arabidopsis thaliana*. Aust. J. Plant Physiol. 19, 427-437.

BAURLE I. & LAUX T. 2003 Apical meristems: the plant's fountain of youth. BioEssays 25, 961-70.

BENFEY P.N. 1999a Is a shoot a root with a view ? Curr. Opin. Plant Biol. 2, 39-43.

BENFEY P.N. 1999b Stem cells: a tale of two kingdoms. Curr. Biol. 9, R171-R172..

BERCETCHE J., CHRIQUI D., LEGAL M. F., NEYRAUD V., LECOCQ F.M. & HALLET J. N. 1993 États nucléaires et dédifférenciation des tissus séminaux de *Pisum sativum* et de *Lupinus albus* L. Acta bot. Gall. 140, 703-706.

BERLETH T. & CHATFIELD S. 2002 Embryogenesis: pattern formation from a single cell. In C. R. Somerville and E. M. Meyerowitz, (eds.) *The Arabidopsis book,* American Society of Plant Biologists, Rockville, MD, http://www.aspb.org/publications/*Arabidopsis*.

BERLETH T. & JÜRGENS G. 1993 The role of the *monopteros* gene in organizing the basal body region of the *Arabidopsis* embryo. Development 118, 575-587.

BERNIER G., KINET J.M. & SACHS R.M. 1981 *The physiology of flowering.* Vol. I and II. CRC Press Inc. Boca Raton, Florida.

BERNIER G., HAVELANGE A., HOUSSA C., PETITJEAN A. & LEJEUNE P. 1993 Physiological signals that induce flowering. Plant Cell 5, 1147-1155.

BERSILLON G. 1955 Recherches sur les Papavéracées. Contribution à l'étude du développement des dicotylédones herbacées. Ann. Sci. Nat. Bot., 11ème série 16, 226-443.

BESNARD-WIBAUT C. 1975 Evolution des synthèses nucléiques dans le méristème caulinaire de l'*Arabidopsis thaliana* L. Heynh. soumis ou non à un traitement induisant la floraison. Etude cytochimique des processus de montaison. Thèse de Doctorat d'Etat, Université Pierre et Marie Curie, Paris, 205 p.

BEVERIDGE C.A., ROSS J.J. & MURFET I.C. 1994 Branching mutant *rms-2* in *Pisum sativum*. Grafting studies and endogenous indole-3-acetic acid levels. Plant Physiol. 104, 953-959.

BHALERAO R.P., EKLOF J., LJUNG K., MARCHANT A., BENNETT M. & SANDBERG G. 2002 Shoot-derived auxin is essential for early lateral root emergence in *Arabidopsis* seedlings. Plant J. 29, 325-332.

BIRNBAUM K. & BENFEY P.N. 2004 Network building: transcriptional circuits in the root. Curr. Opin. Plant Biol. 7, 582-588.

BIRNBAUM K., SHASHA D. E., WANG J. Y., JUNG J. W., LAMBERT G. M., GALBRAITH D. W. & BENFEY P. N. 2003 A gene expression map of the *Arabidopsis* root. Science 302, 1956-1960.

BLILOU I., FRUGIER F., FOLMER S., SERRALBO O., WILLEMSEN V., WOLKENFELT H., ELOY N. B., FERREIRA P. C., WEISBEEK P. & SCHERES B. 2002 The *Arabidopsis* HOBBIT gene encodes a CDC27 homolog that links the plant cell cycle to progression of cell differentiation. Genes Dev. 16, 2566-2575.

BLILOU I., XU J., WILDWATER M., WILLEM-SEN V., PAPONOV I., FRIML J., HEIDSTRA R., AIDA H., PALME K. & SCHERES B. 2005 The PIN protein efflux facilitator network controls growth and patterning in *Arabidopsis* roots. Nature 433, 39-44

BOERJAN W., CERVERA M.T., DELARUE M., BEECKMAN T., DEWITTE W. BELLINI C., CABOCHE M., VAN ONCKELEN H., VAN MONTAGU M. & INZÉ D. 1995 Superroot, a recessive mutation in *Arabidopsis*, confers auxin overproduction. Plant Cell 7, 1405-1419.

BOUCHERON E., GUIVARC'H A., AZMI A., DEWITTE W., VAN ONCKELEN H. & CHRIQUI D. 2001 Competency of *Nicotiana tabacum* L. stem tissues to dedifferentiate is associated with differential levels of cell cycle gene expression and endogenous cytokinins. Planta 215, 267-278.

BOUCHERON E., HEALY J. H., BAJON C., SAUVANET A., REMBUR J., NOIN M., SEKINE M., RIOU-KHAMLICHI C., MURRAY J. A., VAN ONCKELEN H. & CHRIQUI D. 2005 Ectopic expression of *Arabidopsis* CYCD2 and CYCD3 in tobacco has distinct effects on the structural organization of the shoot apical meristem. J. Exp. Bot. 56, 123-134.

BOUDOLF V., VLIEGHE K., BEEMSTER G.T.S., MAGYAR Z., TORRES ACOSTA J.A., MAES S., VAN DER SCHUERE E., INZÉ D. & DE VEYLDER L. 2004 The plant-specific cyclin-dependent kinase CDKB1;1 and transcription factor E2Fa-DPa control the balance of mitotically dividing and endoreduplicating cells in *Arabidopsis*. Plant Cell 16, 2683-2692.

BOWMAN J. L. & ESHED Y. 2000 Formation and maintenance of the shoot apical meristem. Trends Plant Sci. 5, 110-115.

BROSSARD D. 1973 Le bourgeonnement épiphylle chez le *Bryophyllum daigremontianum* Berger (Crassulacées). Etude cytochimique, cytophotométrique et ultrastructurale. Ann. Sc. Nat., Bot., 12ème série 14, 93-214.

BROSSARD D. 1975 La néoformation de bourgeons végétatifs à partir de la moelle du Tabac (*Nicotiana tabacum* L. var. Wisconsin 38) cultivée *in vitro*. Analyse cytochimique, histoautoradiographique et cytophotométrique. Ann. Sc. Nat., Bot., 12ème série 16, 45-150.

BROSSARD D. 1976 Influence de la concentration en kinétine sur l'obtention et le degré de ploïdie des bourgeons néoformés à partir de la moelle de tabac cultivée *in vitro*. Z. Pflanzenphysiol. 78, 323-333.

BROSSARD D. 1978 Microspectrophotometric and ultrastructural analyses of a case of cell differentiation without endopoly-ploidisation: the pith of *Crepis capillaris* L. Wallr. Protoplasma 93, 369-380.

BROSSARD D. 1979 Néoformation de bourgeons végétatifs et inflorescentiels à partir de disques foliaires du *Crepis capillaris* L. Wallr. cultivés *in vitro*. Z. Pflanzenphysiol. 93, 69-81.

BUVAT R. 1989 *Ontogeny, cell differentiation and structure of vascular plants.* Springer Verlag, Berlin, Heidelberg, New York, London, Paris, Tokyo, 581p.

BYRNE M.E., BARLEY R., CURTIS M., ARROYO J.M., DUNHAM M., HUDSON A. & MARTIENSSEN R.A. 2000 Asymmetric leaves 1 mediates leaf patterning and stem cell function in *Arabidopsis*. Nature 408, 21-28.

BYRNE M., TIMMERMANS M., KIDNER C. & MARTIENSSEN R. 2001 Development of leaf shape. Curr. Opin. Plant Biol. 4, 38-43.

CARY A. J., CHE P. & HOWELL S. H. 2002 Developmental events and shoot apical meristem gene expression patterns during shoot development in *Arabidopsis thaliana*. Plant J. 32, 867-877.

CASAMITJANA-MARTINEZ E., HOFHUIS H. F., XU J., LIU C. M., HEIDSTRA R & SCHERES B. 2003 Root-specific CLE19 overexpression and the *sol1/2* suppressors implicate a CLV-like pathway in the control of *Arabidopsis* root meristem maintenance. Curr. Biol.13, 1435-1441.

CASIMIRO I., BEECKMAN T., GRAHAM N., BHALERAO R., ZHANG H., CASERO P., SANDBERG G. & BENNETT M.J. 2003 Dissecting *Arabidopsis* lateral root development. Trends Plant Sci. 8, 1360-1385.

CASIMIRO I., MARCHANT A., BHALERAO R., BEECKMAN T., DHOOGE S., SWARUP R., GRAHAM N., INZÉ D., SANDBERG G., CASERO P. & BENNETT M.J. 2001 Auxin transport promotes *Arabidopsis* lateral root initiation. Plant Cell 13, 843-852.

CATESSON A.M. 1994 Cambial ultrastructure and biochemistry: Changes in relation to vascular tissue differentiation and the seasonal cycle. Int. J. Plant Sci. 155, 251-261

CELENZA J. Jr, GRISAFI P. & FINK G. 1995 A pathway for lateral root formation in *Arabidopsis thaliana*. Genes Dev. 9, 2131-2142.

CHARLTON W.A. 1982 Distribution of lateral root primordia in root tips of *Musa acuminata* Colla. Ann. Bot. 49, 509-520.

CHAUDHURY A.M., LETHAM D.S., CRAIG S. & DENNIS E. S. 1993 amp1: a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. Plant J. 4, 907-916.

CHE P., GINGERICH D.J., LALL S. & HOWELL S.H. 2002 Global and hormone-induced gene expression changes during shoot development in *Arabidopsis*. Plant Cell 14, 2771-2785.

CHEN J.G., ULLAH H., YOUNG J.C., SUSSMAN M.R. & JONES A.M. 2001 ABP1 is required for organized cell elongation and division in *Arabidopsis* embryogenesis. Genes Dev. 15, 902-911.

CHENG J.C., SEELEY K. & SUNG Z.R. 1995 RML1 and RML2 *Arabidopsis* genes required for cell proliferation at the root tip. Plant Physiol. 107, 365-376.

CHOUARD P. & AGHION D. 1961 Modalités de la formation de bourgeons floraux sur des cultures de segments de tige de tabac. C. R. Acad. Sc., Paris 252, 3864-3866.

CHRIQUI D. & BERCETCHE J. 1986 Morphogenetic sequences of the rooting process after *in vitro* culture or *Agrobacterium rhizogenes* inoculation. Giorn. Bot. Ital. 120, 55-68.

CHRIQUI D., DAVID C. & ADAM S. 1988 Effect of the differentiated or dedifferentiated state of tobacco pith tissue on its behaviour after inoculation with *Agrobacterium rhizogenes*. Plant Cell Rep. 7, 111-114.

CHRIQUI D., GUIVARC'H A., DEWITTE W., PRINSEN E. & VAN ONCKELEN H. 1996 Rol genes and root initiation and development. Plant and Soil 187, 47-55.

CHUCK G., LINCOLN C. & HAKE S. 1996 KNAT1 induces lobed leaves with ectopic meristems when overexpressed in *Arabidopsis*. Plant Cell 8, 1277-1289.

CLARK S.E., JACOBSEN S.E., LEVIN J.Z. & MEYEROWITZ E.M. 1996 The CLAVATA and SHOOT MERISTEMLESS loci competitively regulate meristem activity in *Arabidopsis*. Development 122, 1567-1575.

CLARK S.E., RUNNING M.P. & MEYEROWITZ E.M. 1993 CLAVATA: a regulator of meristem and flower development in *Arabidopsis*. Development 119, 397-418.

CLARK S.E., WILLIAMS R. W. & MEYEROWITZ E.M. 1997 The CLAVATA1 gene encodes a putative receptor kinase that control shoot and floral meristem size in *Arabidopsis*. Cell 89, 575-585.

CLINE M.G., 1994 The role of hormones in apical dominance. New approaches to an old problem in plant development. Physiol. Plant. 90, 230-237.

CLOWES F.A.L. 1961 Apical meristems. in: Botanical monographs. Blackwell Scientific, Oxford, 217 pp.

CLOWES F.A.L. 1965 The duration of the G1 phase of the mitotic cycle and its relation to radiosensitivity. New Phytol. 64, 355-359.

COEN E.S. & CARPENTER R. 1993 The metamorphosis of flowers. Plant Cell 5, 1175-1181.

COEN E.S. & MEYEROWITZ E.M. 1991 The war of the whorls: genetic interactions controlling flower development. Nature 353, 31-37.

COLOMBO L., FRANKEN J., KOETJE E., VAN WENT J., DONS H., ANGENENT G. & VAN TUNEN A. 1995 The petunia MADS box gene FBP11 determines ovule identity. Plant Cell 7, 1859-1868.

COTTIGNIES A. 1979 The blockage in the G1 phase of the cell cycle in the dormant shoot apex of ash. Planta 147, 15-19.

D'AMATO F. 1952 Polyploidy in the differentiation and function of tissues and cells in plants. Caryologia 4, 311-358.

DAHL M., MESKIENE I. BÖGRE L., CAMHA D.T., SWOBODA I., HUBMANN R., HIRT H. & HEBERLE-BORS E. 1995 The D-type alfalfa cyclin gene *cyc Ms4* complements G1 cyclin-deficient yeast and is induced in the G1 phase of the cell cycle. Plant Cell 7, 1847-1857.

DAIMON Y., TAKABE K & TASAKA M. 2003 The CUP-SHAPED COTYLEDON genes promote adventitious shoot formation on calli. Plant Cell Physiol. 44, 113-121.

DAWE R.K. & FREELING M. 1991 Cell lineage and its consequences in higher plants. Plant J. 1, 3-8.

DEEBLEY J., STEC A. & HUBBARD L. 1997 The evolution of apical dominance in maize. Nature 386, 485-488.

DE KLERK G.J., VAN DER KRIEKEN W. & DE JONG J.C. 1999 The formation of adventitious roots: new concepts, new possibilities. In Vitro Cell. Dev. Biol. - Plant 35, 189-199.

DELARUE M., PRINSEN E., VAN ONCKELEN H., CABOCHE M. & BELLINI C. 1998 *Sur2* mutations of *Arabidopsis thaliana* define a new locus involved in the control of auxin homeostasis. Plant J. 14, 603-611.

DEN BOER B.G.W. & MURRAY J.A.H. 2000a Triggering the cell cycle in plants. Trends Cell Biol. 10, 245-250.

DEN BOER B.G.W. & MURRAY J.A.H. 2000b Control of plant growth and development through manipulation of cell-cycle genes. Curr. Opin. Biotech. 11, 138-145.

DE VEYLDER L., BEECKMAN T., BEEMSTER G. T. S., DE ALMEIDA ENGLER J., ORMENESE S., MAES S., NAUDTS M., VAN DER SCHUEREN E., JACQMARD A., ENGLER G. & INZÉ D. 2002 Control of proliferation, endoreduplication and differentiation by the *Arabidopsis* E2Fa-DPa transcription factor. EMBO J. 21, 1360-1368.

DE VEYLDER L., JOUBES J. & INZÉ D. 2003 Plant cell cycle transitions. Curr. Opin. Plant Biol. 6, 536-543.

DE VEYLDER L., SEGERS G., GLAB N., CASTEELS P., VAN MONTAGU M. & INZÉ D. 1997 The *Arabidopsis* cks1At protein binds the cyclin-dependent kinases cdc2a At and cdc2b At. FEBS Lett. 412, 446-452.

DEWITTE W., CHIAPPETTA A., AZMI A., STRNAD M., REMBUR J., NOIN M., CHRIQUI D. & VAN ONCKELEN H. 1999 Dynamics of cytokinins in apical shoot meristems of a day neutral tobacco during floral transition and flower formation. Plant Physiol. 119, 111-122.

DEWITTE W. & MURRAY J.A.H. 2003 The plant cell cycle. Annu. Rev. Plant. Biol. 54, 235-264.

DICKINSON T. A. 1978 Epiphylly in Angiosperms. Bot. Rev. 44, 181-232.

DI LAURENZIO L., WYSOCKA-DILLER J., MALAMY J. E., PYSH L., HELARIUTTA Y., FRESHOUR G., HAHN M. G., FELDMANN K. A. & BENFEY P. N. 1996 The SCARECROW gene regulates an asymmetric cell division that *Arabidopsis* for generating the radial organization of the *Arabidopsis* root. Cell 86, 423-433.

DOERNER P. 1998 Root development: quiescent center not so mute after all. Curr. Biol. 8, R42-R44.

DOERNER P., JORGENSEN J. E., YOU R., STEPPUHN J. & LAMB C. 1996 Control of root growth and development by cyclin expression. Nature 360, 520-523.

DOLAN L., JANHAAT K., WILLEMSEN V., LINSTEAD P., POETHIG S., ROBERTS K. & SCHERES B. 1993 Cellular organisation of the *Arabidopsis thaliana* root. Development 119, 71-84.

DOLAN L., DUCKETT C.M., GRIERSON C., LINSTEAD P., SCHNEIDER K., LANSON E., DEAN C., POETHIG S. & ROBERTS K. 1994 Clonal relationships and cell patterning in the root epidermis of *Arabidopsis*. Development 120, 2465-2474.

DOUADY S. & COUDER Y. 1992 Phyllotaxis as a physical self-organized growth process. Phys. Rev. Lett. 68, 2098-2101.

DUCKETT C., OPARKA K., PRIOR D., DOLAN L. & ROBERTS K. 1994 Dye-coupling in the root epidermis of *Arabidopsis* is progressively reduced during development. Development 120, 3247-3255.

ECOLE D. 1970 Premières observations sur la fasciation chez le *Celosia cristata* L. (Amarantacées). C. R. Acad. Sc. Paris 270 , 477-480.

ECOLE D. 1971 Edification de pédoncules sous l'action de l'acide triidobenzoïque chez deux Solanacées: *Lycopersicum esculentum* et *Lycopersicum pimpinellifolium*. C. R. Acad. Sc. Paris. 273, 634-637.

ECOLE D. 1972 Réponse à l'application d'acide 2,3,5 triidobenzoïque (TIBA) chez deux Crassulacées: *Bryophyllum daigremontianum* Berger et *Bryophyllum tubiflorum* Harv. C. R. Acad. Sc. Paris.. 275, 557-560.

ELLIOTT R.E., BETZNER A.S., HUTTNER E., OAKES M.P., TUCKER W.Q.J., GERENTES D., PEREZ P. & SMYTH D.R. 1996 AINTEGUMENTA, an APETALA2-like gene of *Arabidopsis* with pleiotropic roles in ovule development and floral organ growth. Plant Cell 8, 155-168.

ENDRIZZI K., MOUSSIAN B., HAECKER A., LEVIN J.Z. & LAUX T. 1996 The SHOOT MERISTEMLESS gene is required for maintenance of undifferentiated cells in *Arabidopsis* shoot and floral meristems and acts at a different regulatory level than the meristem genes WUSCHEL and ZWILLE. Plant J. 10, 967-979.

ESAU K. 1940 Developmental anatomy of the fleshy storage organ of *Daucus carota*. Hilgardia 13, 175-226.

ESAU K. 1965 *Plant anatomy.* 2nd edition. John Wiley & Sons, Inc. New York, London, 735 p.

ESHED Y., BAUM S.F., PEREA J.V. & BOWMAN J.L. 2001 Establishment of polarity in lateral organs of plants. Curr. Biol. 11, 1251-1260.

ESTRUCH J.J., PRINSEN E., VAN ONCKELEN H., SCHELL J. & SPENA A. 1991 Viviparous leaves produced by somatic activation of an inactive cytokinin-synthesizing gene. Science 254, 1364-1367.

EVANS M.M.S. & BARTON M. K. 1997 Genetics of *Angiosperm* shoot apical meristem development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 673-701.

FELDMAN L.J. & TORREY J.G. 1975 The isolation and culture *in vitro* of the quiescent center of *Zea mays*. Am. J. Bot. 63, 345-355.

FERREIRA P., HEMERLY A., DE ALMEIDA-ENGLER J., VAN MONTAGU M., ENGLER G. & INZÉ D. 1994 Developmental expression of the *Arabidopsis* cycle gene *cyc1At*. Plant Cell 6, 1763-1774.

FISCHER C. & NEUHAUS G. 1996 Influence of auxin on the establishment of bilateral symmetry in monocots. Plant J. 9, 659-669.

FISCHER C., SPETH V., FLEIG-EBERENZ S. & NEUHAUS G. 1997 Induction of zygotic polyembryos in wheat: influence of auxin polar transport. Plant Cell 9, 1767-1780.

FLEMING A.J. 2005 Formation of leaf primordia and phyllotaxy. Curr. Opin. Plant Biol. 8, 53-58.

FLEMING A.J., MANDEL T., HOFMAN S., STERK P., DE VRIES S.C. & KUHLEMEIER C. 1992 Expression pattern of a tobacco lipid transfer protein gene within the shoot apex. Plant J. 2, 855-862.

FLEMING A.J., McQUEEN-MASON S., MANDEL T. & KUHLEMEIER C. 1997 Induction of leaf primordia by the cell wall protein expansin. Science 276, 1415-1418.

FLETCHER J.C., BRAND U., RUNNING M.P., SIMON R. & MEYEROWITZ E.M. 1999 Signaling of cell fate decisions by CLAVATA3 in *Arabidopsis* shoot meristems. Science 283, 1911-1914

FLETCHER J.C. & MEYEROWITZ E.M. 2000 Cell signalling within the shoot meristem. Curr. Opin. Plant Biol. 3, 23-30.

FORSYTH C. & VAN STADEN J. 1994 The effect of root decapitation on lateral root formation and cytokinin production in *Pisum sativum*. Physiol. Plant. 51, 375-379.

FRANCIS D. 1998 Cell size and organ development in higher plant. pp. 187-206 in Francis D., Dudits D. & Inzé D. (eds.) *Plant cell division.* Portland Press Ltd. ISBN 1 85578 089 5.

FRANK M., RUPP H.-M., PRINSEN E., MOTYKA V., VAN ONCKELEN H. & SCHMÜLLING T. 2000 Hormone auto-trophic growth and differentiation identifies mutant lines of

Arabidopsis with altered cytokinin and auxin content and signaling. Plant Physiol. 122, 721-729.

FRIML J., VIETEN A., SAUER M., WEIJERS D., SCHWARZ H., HAMANN T., OFFRINGA R. & JÜRGENS G. 2003 Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. Nature 426, 147-153.

FRUGIS G., GIANNINO D., MELE G., NICOLODI C., INNOCENTI A.M., CHIAPETTA A., BITONTI M.B., DEWITTE W., VAN ONCKELEN H. & MARIOTTI D. 1999 Are homeobox Knotted-like genes and cytokinins the leaf architects? Plant Physiol. 119, 37-37.

GALBRAITH D.W., HARKINS K.R. & KNAPP S. 1991 Systemic endopolyploidy in *Arabidopsis thaliana*. Plant Physiol. 96, 985-989.

GALLOIS J. L., NORA F. R., MIZUKAMI Y. & SABLOWSKI R. 2004 *Wuschel* induces shoot stem cell activity and developmental plasticity in the root meristem. Genes Dev.18, 375-380.

GALWEILER L., GUAN C., MULLER A., WISMAN E., MENDGEN K., YEPHREMOV A. & PALME K. 1998 Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. Science 282, 2226-2230.

GARRISON R. 1955 Studies in the development of axillary buds. Am. J. Bot. 42, 257-266.

GELDNER N., RICHTER S., VIETEN A., MARQUARDT S., TORRES-RUIZ R., MAYER U. & JÜRGENS G. 2003 Partial loss-of-function alleles reveals a role for GNOM in auxin transport-related, post-embryoic development of *Arabidopsis*. Development, 131, 389-400.

GELDNER N., RICHTER S., VIETEN A., MARQUARDT S., TORRES RUIZ R. A., MAYER U. & JÜRGENS G. 2004 Partial loss-of-function alleles reveal a role for GNOM in auxin transport-related, post-embryonic development of *Arabidopsis*. Development 131, 389-400.

GIFFORD E.M. & CORSON G.E. 1971 The shoot apex in seed plants. Bot. Rev. 37, 143-221.

GISEL A., BARELLA S., HEMPEL F.D. & ZAMBRYSKI P.C. 1999 Temporal and spatial regulation of symplastic trafficking during development in *Arabidopsis thaliana* apices. Development 126, 1879-1889.

GIULINI A., WANG J. & JACKSON D. 2004 Control of phyllotaxy by the cytokinin-inducible response regulator ABPHYL1. Nature 430, 1031-1034.

GOLDBERG R.B., DE PAIVA G. & YADEGARI R. 1994 Plant embryogenesis: zygote to seed. Science 266, 605-614.

GOLZ J. F. & HUDSON A. 2002 Signalling in plant lateral organ development. Plant Cell 14 (Suppl.), S277-S288.

GORTER C. J. 1965 Origin of fasciation. Encycl. Plant Physiol. 15, 330-351.

GOTO K., KYOZUKA J. & BOWMAN J. 2001 Turning floral organs into leaves, leaves into floral organs. Genes Dev. 11, 449-456.

GRAFI G. 1998 Cell cycle regulation of DNA replication: the endoreduplication perspective. Exp. Cell Res. 244, 372-378.

GRBIC V. & BLEECKER A. B. 2000 Axillary meristem development in *Arabidopsis thaliana*. Plant J. 21, 215-223.

GREBE M., GADEA J., STEINMANN T., KIENTZ M., RAHFELD J.U., SALCHERT K., KONCZ C. & JÜRGENS G. 2000 A conserved domain of the *Arabidopsis* GNOM protein mediates subunit interaction and cyclophilin 5 binding. Plant Cell 12, 343-56.

GREEN P.B. 1999 Expression of pattern in plants: combining molecular and calculus-based biophysical paradigms. Am. J. Bot. 86, 1059-1076.

GROOT S.P.C., BOUWER R., BUSSCHER M., LINDHOUT P. & DONS H.J. 1995 Increase of endogenous zeatin riboside by introduction of ipt gene in wild type and the lateral suppressor mutant of tomato. Plant Growth Regul. 16, 27-36.

GUILFOYLE T.J., HAGEN G., LI Y., ULSMASOV T., LIU Z.B., STRABALA T. & GEE M.A. 1993 Auxin-regulated transcription. Austr. J. Plant Physiol. 20, 489-502.

GUIVARC'H A., CAISSARD J.C., BROWN S., MARIE D., DE WITTE W., VAN ONCKELEN H. & CHRIQUI D. 1993 Localization of target cells and improvement of *Agrobacterium*-mediated transformation efficiency by direct acetosyringone pretreatment of carrot root discs. Protoplasma 174, 10-18.

GUIVARC'H A., BOCCARA M., PROUTEAU M. & CHRIQUI D. 1999 Instability of phenotype and gene expression in longterm culture of carrot hairy root clones. Plant Cell Rep. 19, 43-50.

GUIVARC'H A., REMBUR J., GOETZ M., ROITSCH T., NOIN M., SCHMÜLLING T. & CHRIQUI D. 2002 Localized expression of the *ipt* gene in the axillary buds of transgenic tobacco (*Nicotiana tabacum* L. cv. SR1) suggests a role for cyto-kinins in tuberization and sink formation. J. Exp. Bot. 53, 1-9.

HACKETT W.P. 1987 Juvenility and maturity. pp.216-231 in: Bonga J.M. & Durzan D.J. (eds.) *Cell and Tissue Culture in Forestry*, vol. 3, Martinus-Mijhoff , Dordrecht, The Netherlands.

HAECKER A., GROB-HARDT R., GEIGES B., SARKAR A., BREUNINGER H., HERRMANN M. & LAUX T. 2004 Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. Development 131, 657-668.

HAGEN G., KLEINSCHMIDT A. & GUILFOYLE T.J. 1984 Auxin-regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. Planta 162, 147-153

HAGEN G., MARTIN G., LI Y., & GUILFOYLE T.J. 1991 Auxin-induced expression of the soybean *gh3* promoter in transgenic tobacco plants. Plant Mol. Biol. 17, 567-579.

HAISSIG B.E. & DAVIS T.D. 1994 A historical evaluation of adventitious rooting research to 1993. pp. 275-331 in: T.D. Davis & B.E. Haissig (eds). *Biology of Adventitious Root Formation*. Plenum Publishing Corp., New York and London

HAMANN T., MAYER U. & JÜRGENS G. 1999 The auxin-insensitive *bodenlos* mutation affects primary root formation and apical-basal patterning in the *Arabidopsis* ambryo. Development 126, 1387-1395.

HANSTEIN J. 1870 Die Entwicklung des Keimes der Monokotylen und der Dikotylen. Bot. Abh 1, 1-112.

HARDTKE C.S. & BERLETH T. 1998 The *Arabidopsis* gene MONOPTEROS encodes a transcription factor mediating embryo axis formation and vascular development. EMBO J. 17, 1405-1411.

HAREVEN D., GUTFINGER T., PAMIS A., ESHED Y. & LIFSCHITZ E. 1996 The making of a compound leaf: genetic manipulation of leaf architecture in tomato. Cell 84, 735-744.

HEALY J.M.S., MENGES M., DOONAN J.H. & MURRAY J.A.H. 2001 The *Arabidopsis* D-type cyclins CycD2 and CycD3 both interact *in vivo* with the PSTAIRE cyclin-dependent kinase Cdc2a but are differentially controlled. J. Biol Chem. 276, 7014-7047.

HELARIUTTA Y., FUKAKI H., WYSOCKA-DILLER J., NAKAJIMA K., JUNG J., SENA G., HAUSER M.T. & BENFEY P.N. 2000 The SHORT-ROOT gene controls radial patterning of the *Arabidopsis* root through radial signaling. Cell 101, 555-567.

HEMERLY A.S., FERREIRA P., DE ALMEIDA ENGLER J., VAN MONTAGU M., ENGLER G. & INZÉ D. 1993 cdc2a expression in *Arabidopsis* is linked with competence for cell division. Plant Cell 5, 1711-1723.

HEPWORTH S.R., VALVERDE F., RAVENSCROFT D., MOURADOV A. & COUPLAND G. 2002 Antagonistic

regulation of flowering-time gene SOC1 by CONSTANS and FLC via separate promoter motifs. EMBO J. 21 16, 4327-4337.

HETZ W., HOCHHOLDINGER F., SCHWALL M. & FEIX G., 1996 Isolation and characterization of *rtcs*, a maize mutant deficient in the formation of nodal roots. Plant J. 10, 845-857.

HEWELT A., PRINSEN E., SCHELL J. VAN ONCKELEN H. & SCHMÜLLING T. 1994 Promoter tagging with a promotorless ipt gene leads to cytokinin-induced phenotypic variability in transgenic tobacco plants: implications of gene dosage effects. Plant J. 6, 879-891.

HIBARA K., TAKADA S. & TASAKA M. 2003 CUC1 gene activates the expression of SAM-related genes to induce adventitious shoot formation. Plant J. 36, 687-96.

HIMANEN K., BOUCHERON E., VANNESTE S., DE ALMEIDA ENGLER J., INZÉ D. & BEECKMAN T. 2002 Auxin-mediated cell cycle activation during early lateral root initiation. Plant Cell , 14, 2339-51.

HOBE M., MULLER R., GRUNEWALD M., BRAND U. & SIMON R. 2003 Loss of CLE40, a protein functionally equivalent to the stem cell restricting signal CLV3, enhances root waving in *Arabidopsis*. Dev Genes Evol. 213, 371-81.

HOFER J., TURNER L., HELLENS R., AMBROSE M., MATTHEWS P., MICHAELS A. & ELLIS N. 1997 UNIFOLIATA regulates leaf and flower morphogenesis in pea. Curr. Biol. 7, 571-587.

HOWARD A. & PELC S.R. 1953 Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Heredity (Suppl.) 5, 216-273.

HOWELL S.H. 1998 *Molecular genetics of plant development.* Cambridge University Press. ISBN 0-521-58784-0. 364 p

HUNTLEY R.P., HEALY S., FREEMAN D., LAVENDER P., DE JAGER S., GREENWOOD J., MAKKER J., WALKER E., JACKMAN M., XIE Q., BANNISTER A.J., KOUZARIDES T., GUTIERREZ C., DOONAN J.H. & MURRAY J.A.H. 1998 The maize retinoblastoma protein homologue ZmRb-1 is regulated during leaf development and displays conserved interactions with G1/S regulators and plant cyclin D (Cyc D) proteins. Plant Mol. Biol. 37, 155-169.

HUNTLEY R.P. & MURRAY J.A.H. 1999 The plant cell cycle. Curr. Opin. Plant Biol. 2, 440-446.

HWANG I. & SHEEN J. 2001 Two-component circuitry in *Arabidopsis* cytokinin signal transduction. Nature 413, 383-389.

INGRAM P.W., GOODRICH J., WILKINSON M.D., SIMON R;, HAUGHN G.W. & COEN E.S. 1995 Parallels between UNUSUAL FLORAL ORGANS and FIMBRIATA genes controlling flower development in *Arabidopsis* and *Antirrhinum*. Plant Cell 7, 1501-1510.

INOUE T., HIGUCHI M., HASHIMOTO Y., SEKI M., KOBAYASHI M., TABATA S., SHINOZAKI S. & KAKIMOTO T. 2001 Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. Nature 409, 1060-1063.

ITOH J.I., HASEGAWA A., KITANO H. & NAGATO Y. 1998 A recessive heterochronic mutation, plastochron1, shortens the plastochron and elongates the vegetative phase in rice. Plant Cell 10, 1511-1521.

JACK T. 2004 Molecular and genetic mechanisms of floral control. Plant Cell 16. S1-17.

JACK T., BROCKMAN L. & MEYEROWITZ E. 1992 The homeotic gene APETALA 3 of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. Cell 68, 683-697.

JACKSON D.P. & HAKE S. 1999 Control of phyllotaxy in maize by the abphy1 gene. Development 126, 315-323.

JACKSON D.P., VEIT B. & HAKE S. 1994 Expression of maize KNOTTED-1 related homeobox genes in the shoot apical meristem predicts pattern of morphogenesis in the vegetative shoot. Development 120, 405-413.

JACQMARD A., DE VEYLDER L., SEGERS G., ENGLER J.D., BERNIER G., VAN MONTAGU M. & INZÉ D. 1999 Expression of *cks1 At* in *Arabidopsis thaliana* indicates a role for the protein in both the mitotic and endoreduplication cycle. Planta 207, 496-504.

JEHAN H., BROWN S., MARIE D., NOIN M., PROUTEAU M. & CHRIQUI D. 1994 Ontogenesis and ploidy levels of plantlets regenerated from *Populus trichocarpa* x *deltoides* cv. Hunnegem root, leaf and stem explants. J. Plant Physiol. 144, 576-585.

JENIK P.D. & IRISH V.F. 2000 Regulation of cell proliferation patterns by homeotic genes during *Arabidopsis* floral development. Development 127, 1267-1276.

JOHN P.C.L. 1998 Cytokinins stimulation of cell division: essential signal transduction is via cdc25 phosphatase. J. Exp. Bot. 49 (Suppl.), 91.

JOHN P.C.L., ZHANG K., DONG C., DIEDERICH L. & WIGHTMAN F. 1993 p34cdc2 related proteins in control of cell cycle progression, the switch between division and differentiation in tissue development, and stimulation of division by auxin and cytokinin. Aust. J. Plant Physiol. 20, 503-526.

JOUBES J., CHEVALIER C., DUDITS D., HEBERLE-BORS E., INZÉ D., UMEDA M. & RENAUDIN J. P. 2000 CDK-related protein kinases in plants. Plant Mol. Biol. 43, 607-620.

JÜRGENS G. 1998 Phases during the life cycle of the flowering plant. In: Westhoff P. *et al.,* eds., *Molecular Plant Development - From Gene to Plant.* Oxford University Press Inc., New York, ISBN 0 19 85 02 03.

JÜRGENS G., TORRES RUIZ R. A. & BERLETH T. 1994 Embryonic pattern formation in flowering plants. Annu. Rev. Genet. 28, 351-371.

KAJIWARA T., FURUTANI M., HIBARA K. & TASAKA M. 2004 The GURKE gene encoding an acetyl-CoA carboxylase is required for partitioning the embryo apex into three subregions in *Arabidopsis*. Plant Cell Physiol. 45, 1122-1128.

KAKIMOTO T. 1996 CKI1, a histidine kinase homolog implicated cytokinin signal transduction. Science 274, 982-985.

KAMIYA N., NAGASAKI H., MORIKAMI A., SATO Y. & MATSOUKA M. 2003 Isolation and characterization of rice WUSCHEL-type homeobox gene that is specifically expressed in the central cells of a quiescent center in the root apical meristem. Plant J. 35, 429-441.

KAPLAN D. 1969 Seed development in *Downingia*. Phyto-morphology 19, 253-278.

KEITH K., KRAML M., DENGLER N.G. & McCOURT P. 1994 *fusca3*: a heterochronic mutation affecting late embryo development in *Arabidopsis*. Plant Cell 6, 589-600.

KELLER B. & LAMB C.J. 1989 Specific expression of a novel cell wall hydroxyproline-rich glycoprotein gene in lateral root initiation. Genes & Dev. 3, 1639-1646.

KERSTETTER R.A. & POETHIG R.S. 1998 The specification of leaf identity during shoot development. Annu. Rev. Cell Dev. Biol. 14, 373-98.

KERSTETTER R.A., BOLLMAN K., TAYLOR R.A., BOMBLIES K. & POETHIG R.S. 2001 KANADI regulates organ polarity in *Arabidopsis*. Nature 411, 706-709.

KESSLER S. & SINHA N. 2004 Shaping up: the genetic control of leaf shape. Curr. Opin. Plant Biol. 7, 65-72.

KIDNER C.A. & MARTIENSSEN R.A. 2004 Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. Nature 438, 81-84.

KING J.J., STIMART D.P., FISCHER R.M. & BLEECKER A.B. 1995 A mutation altering auxin homeostasis and plant morph-ology in *Arabidopsis*. Plant Cell 7, 2023-2037.

KLEE H.J. & ESTELLE H. 1991 Molecular genetic approaches to plant hormone biology. Ann. Rev. Plant Physiol. 42, 529-551.

KOIZUMI K., SUGIYAMA M. & FUKUDA H. 2000 A series of novel mutants of *Arabidopsis thaliana* that are defective in the formation of continuous vascular network: calling the auxin signal flow canalization hypothesis into question. Development 127, 3197-3204.

KOLTUNOV A.M. 1993 Apomixis: embryo sacs and embryos formed without meiosis or fertilization in ovules. Plant Cell 5, 1425-1437.

KONO A., UMEDA-HARA C. , LEE J. , ITO M. , USHIMIYA H. & UMEDA M. 2003 *Arabidopsis* D-type cyclin CycD4;1 is a novel cyclin partner of B2-type cyclin-dependent-kinase. Plant Physiol. 132, 1315-1321.

KUMARAN M.K., BOWMAN J.L. & SUNARESAN V. 2002 YABBY polarity genes mediate the repression of KNOX homeobox genes in *Arabidopsis*. Plant Cell 14, 2761-2770.

KYOZUKA J., KOBAYASHI T., MORITA M. & SHIMAMOTO K. 2000 Spatially and temporally regulated expression of rice MADS box genes with similarity to *Arabidopsis* class A, B and C genes. Plant Cell Physiol. 41, 710-718.

LANCE A. 1957 Recherches cytologiques sur l'évolution de quelques méristèmes apicaux et sur ses variations provoquées par des traitements photopériodiques. Ann. Sc. Nat. Bot., 11ème série 18, 91-421.

LANCE-NOUGARÈDE A. & LOISEAU J.E. 1960 Sur la structure et le fonctionnement du méristème végétatif de quelques Angiospermes aquatiques ou semi-aquatiques dépourvues de moelle. C. R. Acad. Sc. 250, 4438-4440.

LANDRIEU I., DA COSTA M., DE VEYLDER L., DEWITTE F., VANDEPOELE K., HASSAN S., WIERUSZESKI J.M., CORELLOU F., FAURE J.D., VAN MONTAGU M., INZÉ D. & LIPPENS G. 2004a A small CDC25 dual-specificity tyrosine-phosphatase isoform in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 7, 13380-13385.

LANDRIEU I., HASSAN S., SAUTY M., DEWITTE F., WIERUSZESKI J.M., INZÉ D., DE VEYLDER L. & LIPPENS G. 2004b Characterization of the *Arabidopsis thaliana* Arath;CDC25 dual-specificity tyrosine phosphatase. Bioch. Biophys. Res. Commu. 322, 734-739.

LASKOWSKI M.J., WILLIAMS M.E., NUSBAUM M.C. & SUSSEX I.A., 1995 Formation of lateral root meristems is a two-stage process. Development 121, 3303-3310.

LAUFS P., DOCKX J., KRONENBERGER J. & TRAAS J. 1998b MGOUN1 and MGOUN2: two genes required for primordium initiation at the shoot apical and floral meristems in *Arabidopsis thaliana*. Development 125, 1253-1260.

LAUFS P., GRANDJEAN O., JONAK C., KIEU K. & TRAAS J. 1998a Cellular parameters of the shoot apical meristem in *Arabidopsis*. Plant Cell 10, 1375-1389.

LAUFS P., JONAK C. & TRAAS J. 1998c Cells and domains: two views of the shoot meristem in *Arabidopsis*. Plant Physiol. Biochem., 36, 33-45.

LAUFS P., PEAUCELLE A., MORIN H. & TRAAS J. 2004 MicroRNA regulation of the CUC genes is required for boundary size control in *Arabidopsis* meristems. Development 131, 4311-4322.

LAUREYS F., DEWITTE W., WITTERS E., VAN MONTAGU M., INZÉ D. & VAN ONCKELEN H. 1998 Zeatin is indispensable for the G2-M transition in tobacco BY-2 cells. FEBS Lett. 426, 29-32.

LAUX T. 2003 The stem cell concept in plants: a matter of debate. Cell 113, 281-283.

LAUX T.K., MAYER K.F.X., BERGER J. & JÜRGENS G. 1996 The WUSCHEL gene is required for shoot and floral meristem integrity in *Arabidopsis*. Development 122, 87-96.

LEE L., WOLFE D.S., NILSSON O. & WEIGEL D. 1997 A LEAFY co-regulator encoded by UNUSUAL FLORAL ORGANS. Curr. Biol. 7, 95-104.

LEHMAN A., BLACK R. & ECKEN J.R. 1996 Hookless1 an ethylene response gene is required for differential cell elongation in the *Arabidopsis* hypocotyl. Cell 85, 183-194.

LENHARD M. & LAUX T. 1999 Shoot meristem formation and maintenance. Curr. Opin. Plant Biol. 2, 44-50.

LENHARD M. & LAUX T. 2003 Stem cell homeostasis in the *Arabidopsis* shoot meristem is regulated by intercellular movement *opf* CLAVATA3 and its sequestration by CLAVATA1. Development 130, 3163-3173.

LEV-YADUN S. 1995 Differentiation of the ray system in woody plants. Bot. Rev. 61, 45-84.

LEYSER H.M.O. & FURNER I.J. 1992 Characterization of three shoot apical meristem mutants of *Arabidopsis thaliana*. Development 116, 397-403.

LEYSER O., PICKETT F.B., DHARMASIRI S. & ESTELLE M. 1996 Mutations in the AXR3 gene *Arabidopsis* result in altered auxin response including ectopic expression from the SAUR-AC1 promoter. Plant J. 10, 403-413.

LI Y.F., DUBOIS F. & ZHOU D.X. 2001 Ectopic expression of TATA box-binding protein induces shoot proliferation in *Arabidopsis*. FEBS Lett. 489, 187-191

LI Y., HAGEN G. & GUILFOYLE T.J. 1992 Altered morphology in transgenic tobacco plants that overproduce cytokinins in specific tissues and organs. Dev. Biol. 153, 386-395.

LINCOLN C., BRITTON J.H. & ESTELLE M. 1990 Growth and development of the *axr1* mutants of *Arabidopsis*. Plant Cell 2, 1071-1080.

LINCOLN C., LONG J., YAMAGUCHI K., SERKAWA K. & HAKE S. 1994 A *knotted1*-like homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. Plant Cell 6, 1859-1876.

LINTILHAC P.M. & VESECKY T. B. 1980 Mechanical stress and cell wall orientation in plants. I. Photoelastic derivation of principal stresses. With a discussion of the concept of axillarity and the significance of the "arcuate shell zone". Am. J. Bot. 67, 1477-1483.

LIU C.M., XU Z.H. & CHUA N.H. 1993 Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. Plant Cell 5, 621-630.

LOISEAU J.E. 1959 Observation et expérimentation sur la phyllotaxie et le fonctionnement du sommet végétatif chez quelques Balsaminacées. Ann. Sci. Nat. Bot. Sér 11, 201-214.

LONG J.A. & BARTON M.K. 1998 The development of apical embryonic pattern in *Arabidopsis*. Development. 125, 3027-3035.

LONG J.A., MOAN E.I., MEDFORD J.I. & BARTON M.K. 1996 A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of *Arabidopsis*. Nature 379, 66-69.

LOPES M.A. & LARKINS B. A. 1993 Endosperm origin, development and function. Plant Cell 5, 1383-1399.

LOVELL P.L. & WHITE J. 1986 Anatomical changes during adventitious root formation. In: pp. 111-140 in Jackson M.B. (ed.), *New root formation in plants and cuttings*. Martinus Nijhoff Publ., ISBN 90 247 3260 3.

LU P.Z., PORAT R., NADEAU J. A. & OWEILL S.D. 1996 Identification of a meristem L1 layer-specific gene in *Arabidopsis* that is expressed during embryonic pattern

formation and defines a new class of homeobox genes. Plant Cell 8, 2155-2168.

LUCAS W.J., BOUCHÉ-PILLON S., JACKSON D.P., NGUYEN L., BAKER L., DING B. & HAKE S. 1995 Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. Science 270, 1980-1984.

LUND S.T., SMITH A.G. & HACKETT W.P. 1996 Cuttings of a tobacco mutant, rac, undergo cell divisions but do not initiate adventitious roots in response to exogenous auxin. Physiol. Plant. 97, 372-380.

LUND S.T., SMITH A.G. & HACKETT W.P. 1997 Differential gene expression in response to auxin treatment in the wild type and rac, an adventitious rooting-incompetent mutant of tobacco. Physiol. Plant. 114, 1197-1206.

LYNDON R. F. 1990 *Plant development. The cellular basis.* Unwin Hyman LTD Publ. ISBN 0-04-581033-8. 320 p.

LYNN K., FERNANDEZ A., AIDA M., SEDBROOK J., TASAKA M., MASSON P.& BARTON M. K. 1999 The PINHEAD/ZWILLE gene acts pleiotropically in *Arabidopsis* development and has overlapping functions with the ARGONAUTE gene. Development 126, 469-481.

MAGYAR Z., MESZAROS T., MISKOLSZI P., DEAK M., FEHER A., BROWN S., KONDOROSI E., ATHANASIADIS A., PONGOR S., BILGIN M., BAKO L., KONCZ C. & DUDITS D. 1997 Cell cycle phase specificity of putative cyclin-dependent kinase in synchronized alfafa cells. Plant Cell 9, 223-235.

MÄHÖNEN A.P., BONKE M., KAUPPINEN L., RIIKONEN M., BENFEY P.N. & HELARIUTTA Y. 2000 A novel two component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root. Genes Dev. 14, 2938-2943.

MALAMY J.E., BENFEY P.N. 1997 Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. Development 124, 33-44.

MALAYER J.C. & GUARD A.T. 1964 A comparative developmental study of the mutant sideshootless and normal tomato plants. Am. J. Bot. 51, 140-143.

MANDEL M.C., GUSTAFSON-BROWN C., SAVIDGE B. & YANOFSKI M.F. 1992 Molecular characterization of the *Arabidopsis* floral homeotic gene APETALA 1. Nature 360, 273-277

MARCOTRIGIANO M. & GOUIN F.R. 1984 Experimentally synthesized plant chimeras 1. *In vitro* recovery of *Nicotiana tabacum* L. chimeras from mixed callus cultures. Ann. Bot. 54, 503-511.

MAR CASTELLANO M. & SABLOWSKI R. 2005 Intercellular signalling in the transition from stem cells to organogenesis in meristems. Curr. Opin. Plant Biol. 8, 26-31.

MANDEL M.A. & YANOFSKY M.F. 1995a A gene triggering flower development in *Arabidopsis*. Nature 377, 522-524.

MANDEL M.A. & YANOFSKY M.F. 1995b The *Arabidopsis* MADS box gene is expressed in inflorescence meristems and is negatively regulated by APETALA 1. Plant Cell 7, 1763-1771.

MARC J. & HACKETT W.P. 1991 Gibberellin-induced reorganization of spatial relationship of emerging leaf primordia at the shoot apical meristem of *Hedera helix* L. Planta 185, 171-178.

MARTINEZ M.C., JRGENSEN J.E., LAWTON M.A., LAMB C.J. & DOERNER P.N. 1992 Spatial pattern of cdc2 expression in relation to meristem activity and cell proliferation during plant development. Proc. Natl. Acad. Sci. USA 89, 7360-7364.

MASUCCI J.D., RERIE W.G., FOREMAN D.R., ZHANG M., GALWAY M.E., MARKS M.D. SCHIEFELBEIN J.W. 1996 The homeobox gene GLABRA2 is required for position dependent cell differentiation in the root epidermis of *Arabidopsis thaliana*. Development 122, 1253-1260.

MAYER U., BÜTTNER G. & JÜRGENS G. 1993 Apical-basal pattern formation in the *Arabidopsis* embryo. Studies on the role of the gnom gene. Development 117, 149-162.

MAYER K.F.X., SCHOOF H., HAECKER A., LENHARD M., JÜRGENS G. & LAUX T.K. 1998 Role of WUSCHEL in regulating cell fate in the *Arabidopsis* shoot meristem. Cell 95, 805-815.

McCONNELL J.R. & BARTON M.K. 1995 Effect of mutations in the PINHEAD gene of *Arabidopsis* on the formation of shoot apical meristems. Dev. Gen. 16, 358-366.

McCONNELL J.R. & BARTON M.K. 1998 Leaf polarity and meristem formation in *Arabidopsis*. Development 125, 2935-2942.

McCULLY M. 1975 The development of lateral roots. pp. 105-124 in Torrey J.G. and Clarkson D.T. (eds.). *The development and function of roots*. Academic. Press London, New York, San Francisco. ISBN 0 12 6957 50 9.

McHALE N.A. & KONING R.E. 2004 MicroRNA-directed cleavage of *Nicotiana sylvestris* PHAVOLUTA mRNA regulates the vascular cambium and structure of apical meristems. Plant Cell. 16, 1730-1740.

McKIBBIN R.S., HALFORD N.G. & FRANCIS D. 1998 Expression of fusion yeast *cdc25* alters the frequency of lateral root formation in transgenic tobacco. Plant Mol. Biol. 36, 601-612.

MEDFORD J.I., MORGAN R., El-SAWI Z. & KLEE H.J. 1989 Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. Plant Cell 1, 403-413.

MEINKE D.W., 1992 A homeotic mutant of *Arabidopsis* with leafy cotyledons. Science 258, 1647-1650.

MELARAGNO J.E., MEHROTRA B. & COLEMAN A.W. 1993 Relationship between endopolyploidy and cell size in epidermal tissue of *Arabidopsis*. Plant Cell 5, 1661-1668.

MELLEROWICZ E.J., RIDING R.T. & LITTLE C.H.A. 1989 Genomic variability in the vascular cambium of *Abies balsamina*. Can. J. Bot. 67, 990-996.

MENGES M., HENNIG L., GRUISSEM W. & MURRAY J.A.H. 2002 Cell cycle-regulated gene expression in *Arabidopsis*. J. Biol. Chem. 277, 41987-42002.

MENGES M., DE JAGER S.M., GRUISSEM W. & MURRAY J.A.H. 2005 Global analysis of the corer cell cycle regulators of *Arabidopsis* identifies novel genes, reveals multiple and highly specific profiles of expression and provides a coherent model for plant cell cycle control. Plant J. 41, 546-566.

MENGES M. & MURRAY J.A.H. 2002 Synchronous *Arabidopsis* suspensions cultures for analysis of cell-cycle gene activity. Plant J. 30, 203-212.

MENGES M., HENNIG L., GRUISSEM W. & MURRAY J.A.H. 2003 Genome-wide gene expression in an *Arabidopsis* cell suspension. Plant Mol. Biol. 56, 423-442.

MEYEROWITZ E.M., 1997 Genetic control of cell division patterns in developing plants. Cell 88, 299-308.

MICHAELS S.D., HIMELBLAU E., KIM S.Y., SCHOMBURG F.M. & AMASINO R.M. 2005 Integration of flowering signals in winter-annual *Arabidopsis*. Plant Physiol. 137, 149-156.

MILLER M.B. & LYNDON R.F. 1975 The cell cycle in vegetative and floral shoot meristems measured by a double labeling technique. Planta 126, 37-43.

MIRONOV V., DE VEYLDER L., VAN MONTAGU M. & INZÉ D. 1999 Cyclin-dependent kinases and cell division in plants - The nexus. Plant Cell 11, 440-446

MORDHORST A.P., TOONEN M.A. & DE VRIES S. 1997 Plant embryogenesis. Crit. Rev. Plant Sci. 16, 535-576.

MORDHORST A. M., VOERMAN K.J., MARTOG M.V., MEISER E.A., VAN WENT J., KOORNNEEF M. & DE VRIES

S.C. 1998 Somatic embryogenesis in *Arabidopsis thaliana* is facilitated by mutations in genes repressing meristematic cell divisions. Genetics 149, 549-563.

MORRIS R. O. 1997 Hormonal regulation of seed development. In: *Cellular and molecular biology of plant seed development*. B.A. Larkino and I.K. Vasil (eds). Kluwer Acad. Publ., pp. 117-149. ISBN 0-7923-4645-9.

MOURADOV A., CREMER F. & COUPLAND G. 2002 Control of flowering time: interacting pathways as a basis for diversity. Plant Cell 14, S111-S130.

MOUSSIAN B., SCHOOF H., HAECKER A., JÜRGENS G. & LAUX T. 1998 Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during *Arabidopsis* embryogenesis. EMBO J. 17, 1799-1809.

MUDAY G.K. & HAWORTH P. 1994 Tomato root growth, gravitropism, and lateral development: correlation with auxin transport. Plant Physiol. Biochem. 32, 193-203.

MURRAY B.E. 1957 The ontogeny of adventitious stems on roots of creeping-rooted alfalfa. Can. J. Bot. 35, 463-475.

NAGL W. 1979 Differential DNA replication in plants: a critical review. Z. Pflanzenphysiol. 95, 283-314.

NAKAJIMA K., SENA G., NAWY T. & BENFEY P. N. 2001 Intercellular movement of the putative transcription factor SHR in root patterning. Nature 413, 307-311.

NAPOLI C.A., BEVERIDGE C.A. & SNOWDEN K.C. 1999 Re-evaluating concepts of apical dominance and the control of axillary bud outgrowth. Curr. Top. Dev. Biol. 44, 127-167.

NISHIMURA A., TAMAOKI M., SATO Y. & MATSUOKA M. 1999 The expression of tobacco *knotted1*-type class 1 homeobox genes correspond to regions predicted by the cytohistological zonation model. Plant J. 18, 337-347.

NITSCH J.P. 1969 Experimental androgenesis in *Nicotiana*. Phytomorphology 19, 389-404.

NOUGARÈDE A. 1967 Experimental cytology of the shoot apical cells during vegetative growth and flowering. Intern. Rev. Cytol. 21, 203-351.

NOUGARÈDE A. & REMBUR J., 1977 Determination of cell cycle and DNA synthesis duration in the shoot apex of *Chrysanthemum segetum* L. by double-labelling autoradiographic techniques. Z. Pflanzenphysiol. 85, 341-356.

NOUGARÈDE A. & REMBUR J. 1978 Variations of the cell cycle phases in the shoot apex of *Chrysanthemum segetum* L. Z. Pflanzenphysiol. 90, 379-389.

NOUGARÈDE A. & REMBUR J. 1985 Le point végétatif en tant que modèle pour l'étude du cycle cellulaire et de ses points de contrôle. Bull. Soc. Bot. Fr., 132, Actual. Bot. 9-34.

NOUGAREDE A. & RONDET P. 1975 Index mitotiques et teneurs en DNA nucléaire du méristème axillaire de la feuille de rang 6 et du point végétatif terminal, chez le *Pisum sativum* L. var. nain hâtif d'Annonay. C. R. Acad. Sc., série D 280, 709-712.

NOUGARÈDE A., REMBUR J. & RONDET P. 1981 Réactivation du bourgeon cotylédonaire du pois en réponse à la kinétine. Can. J. Bot. 59, 590-603.

NUTI RONCHI V., BENNICI A. & MARTINI G. 1973 Nuclear fragmentation in dedifferentiating cells of *Nicotiana glauca* pith tissue grown *in vitro*. Cell Diff. 2, 77-86.

ORI N., ESHED Y., CHUCK G., BOWMAN J.L. & HAKE S. 2000 Mechanisms that control *knox* gene expression in the *Arabidopsis* shoot. Development 127, 5523-5532.

ORMENESE S., DE ALMEIDA ENGLER J., DE GROOT R., DE VEYLDER L., INZÉ D. & JACQMARD A. 2004 Analysis of the spatial expression pattern of seven Kip related proteins (KRPs) in the shoot apex of *Arabidopsis thaliana*. Ann. Bot. 93, 575-580.

OTSUGA D., DE GUZMAN B., PRIGGE M.J., DREWS G.N. & CLARK S. 2001 REVOLUTA regulates meristem initiation at lateral positions. Plant J. 25, 223-236.

PARCY F., NILSSON O., BUSH M.A., LEE I. & WEIGEL D. 1998 A genetic framework for floral patterning. Nature 395, 561-566.

PAULET P. & NITSCH J.P. 1964 Néoformation de fleurs *in vitro* sur des cultures de tissus de racines de *Cichorium intybus* L. C. R. Acad. Sc., Paris 258, 5952-5955.

PELAZ S., TAPIA-LOPEZ R., ALVAREZ-BUYLLA E.R. & YANOFSKY M.F. 2001 Conversion of leaves into petals in *Arabidopsis*. Curr. Biol. 11, 182-184.

PENNELL R.I., CRONK Q.C.B., FORSBERG L.S., STÖHR C., SNOGERUP L., KJELLBOM P. & McCABE P., 1995, Cell-context signalling. Phil. Trans. Roy. Soc. Lond. B, 350, 87-93.

PETERSON R.L. 1975 The initiation and development of root buds. pp. 126-161 in Torrey J.G. & Clarkson D.T. (eds.). *The development and function of roots*. Acad. Press, London, New York, San Francisco. ISBN 0 12 6957 50 9.

PETERSON R.L. & PETERSON C.A. 1986 Ontogeny and anatomy of lateral roots. pp. 1-30, in Jackson M.B. (ed.) *New root formation in plants and cuttings*. Martinus Nijhoff Publ. ISBN 90 247 3260 3.

PIEN S., WYRZYKOWSKA J., Mc QUEEN-MASON S., SMART C. & FLEMING A. 2001 Local expression of expansin induces the entire process of leaf development and modifies leaf shape. Proc. Natl. Acad. Sci. USA 98, 11812-11817.

PLANTEFOL L. 1947 Hélices foliaires, point végétatif et stèle chez les dicotylédones. La notion d'anneau initial. Rev. Gén. Bot. 54, 49-81.

POLLOCK E.G. & JENSEN W.A. 1964 Cell development during early embryogenesis in *Capsella* and *Gossypium*. Am. J. Bot. 51, 915-921.

PRINSEN E., BERCETCHE J., CHRIQUI D. & VAN ONCKELEN H. 1992 *Pisum sativum* epicotyls inoculated with *Agrobacterium rhizogenes* agropine strains harboring various T-DNA fragments: morphology, histology, and endogenous indole-3-acetic acid and indole-3-acetamide content. J. Plant Physiol. 140, 75-83.

PROJETTI M.L. & CHRIQUI D. 1986a Totipotence du péricycle des organes souterrains et aériens du *Rorippa sylvestris* Bess. I - Rhizogenèse et caulogenèse à partir de racines en place ou cultivées *in vitro*. Can. J. Bot. 64, 1760-1769.

PROJETTI M.L. & CHRIQUI D. 1986b Totipotence du péricycle des organes souterrains et aériens du *Rorippa sylvestris* Bess. II - Régénération à partir de feuilles détachées ou de fragments foliaires cultivés *in vitro*. Can. J. Bot. 64, 1770-1777.

REINHARDT D., MANDEL T. & REINHARDT D. 2000 Auxin regulates the initiation and radial position of plant lateral organs. Plant Cell 12, 507-518.

REINHARDT D., PESCE E. R., STIEGER P., MANDEL T., BALTENSPERGER K., BENNETT M., TRAAS J., FRIML J. & KUHLEMEIER C. 2003 Regulation of phyllotaxis by polar auxin transport. Nature 426, 255-260.

REINHARDT D., WITTWER F., MANDEL T. & KUHLEMEIER C. 1998 Localized upregulation of a new expansin gene predicts the site of leaf formation in the tomato meristem. Plant Cell 10, 1427-1437.

REMBUR J. 1972 Etude cytophotométrique des variations des teneurs en DNA au cours des premières heures de la germination chez le *Xanthium pennsylvanicum* Wallr. C. R. Acad. Sc. 275, 903-906.

RINNE P.L. & VAN DER SCHOOT C. 1998 Symplasmic fields in the tunica of the shoot apical meristem coordinate morphogenetic events. Development 125, 1477-1485.

RIOU-KHAMLICHI C., HUNTLEY R., JACQMARD A. & MURRAY J.A.H. 1999 Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. Science 283, 1541-1544.

RIOU-KHAMLICHI C., MENGES M., HEALY J.M.S. & MURRAY J.A.H. 2000 Sugar control of the plant cell cycle: differential regulation of *Arabidopsis* D-type cyclin gene expression. Mol. Cell Biol. 20, 4513-4521.

RONDET P. 1965 Mise en place des méristèmes chez les Angiospermes au cours de l'embryogenèse. Bull. Soc. Fr. Physiol. Vég. 11, 175-183.

RUIZ-MEDRANO R., XOCONOSTLE-CAZARES B. & LUCAS W.J. 1999 Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. Development 126, 4405-4419.

RUPP R.M., FRANK M., WERNER T., STRNAD M. & SCHMÜLLING T. 1999 Increased steady state mRNA levels of the STM and KNAT1 homeoboxes genes in cytokinin over-producing *Arabidopsis thaliana* indicate a role for cytokinins in the shoot apical meristem. Plant J. 18, 557-567.

SABATINI S., BEIS D., WOLKENFELT H., MURFETT J., GUILFOYLE T., MALAMY J., BENFEY P., LEYSER O., BECHTOLD N., WEISBEEK P. & SCHERES B. 1999 An auxin-dependent distal organizer of pattern and polarity in the *Arabidopsis* root. Cell 99, 463-472.

SAINT-CÔME R. 1985 Cycles cellulaires dans le méristème caulinaire de *Coleus blumei* Benth. de la germination à la floraison. Aspects morphogénétiques et cytophysiologiques. Thèse de Doctorat d'Etat, Université P. M. Curie, 300 pp.

SATINA S., BLAKESLEE A.F. & AVERY A. 1940 Demonstration of three germ layers in the shoot apex of *Datura* by means of induced polyploidy in periclinal chimeras. Am. J. Bot. 27, 895-907.

SATTLER R. 1988 Homeosis in plants. Am. J. Bot. 75, 1606-1617.

SATTLER R. & MAIER U. 1977 Development of epiphyllous appendages of *Begonia hispida* var. *cuculifera*: implications or comparative morphology. Can. J. Bot. 55, 411-425.

SAUER E., VAN HOUWELINGEN A., KLOOS D., MOL J. & KOES R. 1996 The no apical meristem gene of *Petunia* is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell 85, 159-179.

SAUTER M. 1997 Differential expression of a CAK (*cdc2* - activating kinase)-like protein kinase, cyclins and *cdc2* genes from rice during the cell cycle and in response to gibberellin. Plant J. 11, 181-190.

SAWA S., WATANABE K., GOTO K., KANAYA E., MORITA E.H. & OKADA K. 1999 FILAMENTOUS FLOWER, a meristem and organ identity gene of *Arabidopsis*, encodes a protein with a zinc finger and HMG-related domains. Genes Dev. 13, 1079-1088.

SCANLON M.J. 2003 The polar auxin transport inhibitor N-1-naphthylphthalamic acid disrupts leaf initiation, KNOX protein regulation, and formation of leaf margins in maize. Plant Physiol. 133, 597-605.

SCHERES B. & BERLETH T. 1998 Root development: new meanings for root canal? Curr. Opin. Plant Biol. 1, 32-36.

SCHERES B., DI LAURENZIO L., WILLEMSEN V., HAUSER M. T., JANMAAT K., WEISBEEK P. & BENFEY P. N. 1995 Mutations affecting the radial organisation of the *Arabidopsis* root display specific defects throughout the embryonic axis. Development 121, 53-62.

SCHERES B., McKAHN H.I. & VAN DEN BERG C. 1996 Roots redefined: anatomical and genetic analysis of root development. Plant Physiol. 111, 959-964.

SCHERES B., WOLKENFELT H., WILLEMSEN V., TERLOUW M., LANSON E., DEAN C. & WEISBEEK P. 1994 Embryonic origin of the *Arabidopsis* primary root and root meristem initials. Development 120, 2475-2487.

SCHICHNES D., SCHNEEBERGER R. & FREELING M. 1997 Induction of leaves directly from leaves in the maize mutant *Lax midrib1-0*. Dev. Biol. 186, 36-45.

SCHIEFELBEIN J.W., MASUCCI J.D. & WANG H. 1997 Building a root. The control of patterning and morphogenesis during root development. Plant Cell 9, 1089-1098.

SCHNEEBERGER R.G., BECRAFT P.W., HAKE S. & FREELING H. 1995 Ectopic expression of the KNOX homeobox gene ROUGH SHEATH 1 alters cell fate in the maize leaf. Genes Dev. 9, 2292-2304.

SCHOOF H., LENHARD M., HAECKER A., MAYER K.F.X., JÜRGENS G. & LAUX T.K. 2000 The stem cell population of *Arabidopsis* shoot meristem is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. Cell 100, 635-644.

SCHRICK K., MAYER U., HORRICHS A., KUHNT C., BELLINI C., DANGL J., SCHMIDT J. & JÜRGENS G. 2000 FACKEL is a sterol C-14 reductase required for organized cell expansion in *Arabidopsis* embryogenesis. Genes Dev. 14, 1471-1484.

SCHULZ S.R. & JENSEN W. 1968 *Capsella* embryogenesis: the early embryo. J. Ultrastruct. Res. 22, 376-392.

SCHRADER J., BABA K., MAY S.T., PALME K., BENNET M., BHALE RAO R.P. & SANDBERG G. 2003 Polar auxin transport in the wood forming tissues of hybrid aspen is under simultaneous control of developmental and environmental signals. Proc. Natl. Acad. Sci. USA 100, 10096-10101.

SCHUMACHER K., SCHMITT T., ROSSBERG M., SCHMITZ G. & THERES K. 1999 The Lateral suppressor (*Ls*) gene of tomato encodes a new member of the VHIID protein family. Proc. Natl. Acad. Sci. USA 96, 290-295.

SCHWABE W.W. 1984 Phyllotaxis. pp 403-440 in Barlow P.W. & Carr, D.J. (eds,) *Positional Controls of Plant Development* Cambridge University Press.

SELKER J.M.L. & LYNDON R.F. 1996 Leaf initiation and *de novo* pattern formation in the absence of an apical meristem and preexisting patterned leaves in watercress (*Nasturtium officinale*) axillary explants. Can. J. Bot. 74, 625-641.

SENA G., JUNG J.W. & BENFEY P.N. 2004 A broad competence to respond to SHORT ROOT revealed by tissue-specific ectopic expression. Development 131, 2817-2826.

SENTOKU N., YUTAKA S., KURATA N., ITO Y., KITANO H. & MATSUOKA M. 1999 Regional expression of the rice KNI-type homeobox gene family during embryo, shoot and flower development. Plant Cell 11, 1651-1665.

SESSIONS A., WEIGEL D. & YANOFSKI M.F. 1999 The *Arabidopsis thaliana* MERISTEM LAYER1 promoter specifies epidermal expression in meristems and young primordia. Plant J. 20, 259-263.

SHAH J.J. & PATEL J.D. 1972 The shell zone: its identification and probable function in some dicotyledons. Am. J. Bot. 59, 683-690.

SHAHAR T., HENNIG N., GUTFINGER T., HAREVEN D. & LUFTSCHITZ E. 1992 The tomato *66.3-kD* polyphenoloxidase gene: molecular identification and developmental expression. Plant Cell 4, 135-147.

SHEN W.H. 2002 The plant *E2F-Rb* pathway and epigenetic control. Trends Plant Sci. 7, 505-511.

SHINDO S., ITO M., VEDA K., KATO M. & HASEBE M. 1999 Characterization of MADS genes in the gymnosperm *Gnetum parvifolium* and its implication on the evolution of reproductive organs in seed plants. Evol. Dev. 1, 1-11.

SIEGFRIED K.R., ESHED Y., BAUM S.F., OTSUGA D., DREWS G.N. & BOWMAN J. 1999 Members of the YABBY gene family specify abaxial cell fate in *Arabidopsis*. Development 126, 4117-4128.

SINHA N.R., WILLIAMS R.E. & HAKE S. 1993 Overexpression of the maize homeobox gene KNOTTED-1 causes a switch from determinate to indeterminate cell fates. Genes Dev., 7 787-795.

SKOOG F. & MILLER C.O. 1957 Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp. Soc. Exp. Biol. 11, 118-131.

SMIGUCKI A.C. & OWENS L.D. 1989 Cytokinin-to-auxin ratios and morphology of shoots and tissues transformed by a chimeric isopentenyl transferase gene. Plant Physiol. 91, 808-811.

SMITH D.L. & FEDOROFF N.V. 1995 LRP1, a gene expressed in lateral and adventitious primordia of *Arabidopsis*. Plant Cell 7, 735-745.

SMITH L. G. & HAKE S. 1995 The initiation and determination of leaves. Plant Cell 4, 1017-1027.

SMITH L.G., JACKSON D. & HAKE S. 1995 Expression of knotted1 marks shoot meristem formation during maize embryogenesis. Dev. Genet. 16, 344-348.

SONI R., CARMICHAEL J.P., SHAH Z.M. & MURRAY J.A.H. 1995 A family of cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. Plant Cell 7, 85-103.

SORRELL D.A., COMBETTES B., CHAUBET-GIGOT N., GIGOT C. & MURRAY J.A.H. 1999 Distinct cyclin D genes show mitotic accumulation or constant levels of transcripts in tobacco Bright Yellow-2 cells. Plant Physiol. 119, 343-351.

SOSSOUNTZOV L., MALDINEY R., SOTTA B., SABBAGH I., HABRICOT Y., BONNET M. & MIGINIAC E. 1988 Immunocytochemical localization of cytokinins in *Craigella* tomato and a sideshootless mutant. Planta 175, 291-304.

SPURR A.R. 1949 Histogenesis and organization of the embryo in *Pinus strobus* L. Am. J. Bot. 36, 629-641.

STAFSTROM J.P., 1995 Developmental potential of shoot buds. pp. 257-279 in Gartner B.L. (ed.) *Plant stems*. Acad. Press,. ISBN 0-12-276460-9.

STEEVES T.A. & SUSSEX I.M. 1989 *Patterns in Plant Development* (2nd edition). Cambridge Univ. Press.

STEINMANN T., GELDNER N., GREBE M., MANGOLD S., JACKSON C.L., PARIS S., GÄLWEILER L., PALME K. & JÜRGENS G. 1999 Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. Science 286, 316-318.

STERK P., BOOIJ H., SCHELLEKENS G.A., VAN KAMMEN A. & DE VRIES S.C. 1991 Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell 3, 907-921.

STEWART R.N. 1978 Ontogeny of the primary body in chimeral forms of higher plants. pp.131-160 in Sublelny S. & Sussex I.M. (eds.) *The Clonal Basis of Development*. Academic Press, New York.

STEWART R.N. & DERMEN H. 1975 Flexibility in ontogeny as shown by the contribution of the shoot apical layers to leaves of periclinal chimeras. Am. J. Bot. 62, 935-947.

STEWART R.N., MEYER F.G. & DERMEN H. 1972 *Camellia* + “Daisy Eagleson”, a graft chimera of *Camellia sasanqua* and *C. japonica*. Am. J. Bot. 59, 515-524.

STIRNBERG. P., CHATFIELD S. P. & LEYSER H.M.O. 1999 AXR1 acts after lateral bud formation to inhibit lateral bud growth in *Arabidopsis*. Plant Physiol. 121, 839-847.

STIRNBERG. P., VAN DE SANDE K. & LEYSER H.M.O. 2002 MAX1 and MAX2 control shoot lateral branching in *Arabidopsis*. Development 129, 1131-1141.

STONE S.L., KWONG L.W., YEE K.M., PELLETIER J., LEPINIEC L., FISCHER R.L., GOLDBERG R.B. & HARADA J.J. 2001 LEAFY COTYLEDON 2 encodes a B3 domain transcription factor that induces embryo development. Proc. Natl. Acad. Sci. USA 98, 11806-11811.

SUGIMOTO-SHIRASU K. & ROBERTS K. 2003 “Big it up”: endoreduplication and cell-size control in plants. Curr. Opin. Plant Biol. 6, 544-553.

SUN Y., DILKES B.P., ZHANG C., DANTE R.A., CARNEIRO N.P., LOWE K.S., JUNG R., GORDON-KAMM W. J. & LARKINS B.A. 1999 Characterization of maize (*Zea mays* L.) *Wee1* and its activity in developing endosperm. Proc. Natl. Acad. Sci. USA 30, 4180-4185.

SUNDBERG B., ERICSSON A., LITTLE C.H.A., NASHOLM T. & GREF R. 1993 The relationship between crown size and ring width in *Pinus sylvestris* L. stems: dependence on indole-3-acetic acid, carbohydrates and nitrogen in the cambial region. Tree Physiol. 12, 347-362.

SUSSEX I.M. 1955 Morphogenesis in *Solanum tuberosum* L. Experimental investigation of leaf dorsiventrality and orientation in the juvenile shoot. Phytomorphology 5, 286-300.

SZYMKOWIAK E.J. & SUSSEX I.M. 1993 Effects of lateral suppressor on petal initiation in tomato. Plant J. 4, 1-7.

SZYMKOVIAK E.J. & SUSSEX I.M., 1996 What chimeras can tell us about plant development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 351-376.

TAKADA S., HIBARA K.I., ISHIDA T. & TASAKA M. 2001 The CUP-SHAPED COTYLEDON1 gene of *Arabidopsis* regulates shoot apical meristem formation. Development 128, 1127-1135.

TALBERT P.B., ALDER H.T., PARKS D.W. & COMAI L. 1995 The REVOLUTA gene is necessary for apical meristem development and for limiting cell divisions in the leaves and stems of *Arabidopsis thaliana*. Development 121, 2723-2735.

TANTIKANJANA T., YONG J.W.H., LETHAM D.S., GRIFFITH M., HUSSAIN M., LJUNG K., SANDBERG G. & SUNDARESAN V. 2001 Control of axillary bud initiation and shoot architecture in *Arabidopsis* through the *supershoot* gene. Genes Dev. 15, 1577-1588.

TAYLOR R.L. 1967 The foliar embryos of *Malaxis paludosa*. Can. J. Bot. 45, 1553-1556.

TAYLOR B.H. & SCHEURING C.F. 1994 A molecular marker for lateral root initiation: the RSI-1 gene of tomato (*Lycopersicum esculentum* Mill) is activated in early lateral root primordia. Mol. Gen. Genet. 243, 148-157.

THOMA S., HECHT U., KIPPERS A., BOTELLA J., DE VRIES S. & SOMERVILLE C. 1994 Tissue-specific expression of a gene encoding a cell wall-localized lipid transfer protein from *Arabidopsis*. Plant Physiol. 105, 35-45.

TIAN H. C. & MARCOTRIGIANO H. 1993 Origin and development of adventitious shoot meristems initiated on plant chimeras. Dev. Biol. 155, 259-269.

TOPPING J., WEI N. & LINDSEY K. 1991 Functional tagging of regulatory elements in the plant genome. Development 112, 1009-1019.

TORRES ACOSTA J.A., DE ALMEIDA ENGLER J., RAES J., MAGYAR Z., DE GROOT R., INZÉ D. & DE VEYLDER L. 2004 Molecular characterization of *Arabidopsis* PHO-80-like proteins, a novel class of CDKA;1-interacting cyclins. Cell Mol. Life Sci. 61, 1485-1497.

TRAAS J., BELLINI C., NACRY P., KRONENBERGER J., BOUCHEZ D. & CABOCHE M. 1995 Normal differentiation patterns in plants lacking microtubular preprophase bands. Nature 375, 676-677.

TSIANTIS M., SCHNEEBERGER R., GOLZ J.F., FREELING M. & LANGDALE J.A. 1999 The maize ROUGH SHEATH 2 gene and leaf development programs in monocot and dicot plants. Science 284, 154-156.

VAN DEN BERG C., WILLEMSEN V., HAGE W., WEISBEEK P. & SCHERES B. 1995 Cell fate in the *Arabidopsis* root meristem determined by directional signalling. Nature 378, 62-65.

VANDEPOELE K., RAES J., DE VEYLDER L., ROUZE P., ROMBAUTS S. & INZÉ D. 2002 Genome-wide analysis of core cell cycle genes in *Arabidopsis*. Plant Cell 14, 903-916.

VAN 'T HOF J. 1973 The regulation of cell division in higher plants. Brookhaven Symp. 25, 152-165.

VERA P., LAMB C.J. & DOERNER P.W. 1994 Cell-cycle regulation of hydroxyproline-rich glycoprotein HRGPnt3 gene expression during the initiation of lateral root meristems. Plant J. 6, 717-727.

VERNOUX T., KRONENBERGER J., GRANDJEAN O., LAUFS P. & TRAAS J. 2000a PIN-FORMED1 regulates cell fate at the periphery of the shoot apical meristem. Development 127, 5157-5165.

VERNOUX T., WILSON R.C., SEELEY K.A., REICHELD J.P., MUROY S., BROWN S., MAUGHAM S., COBBETT C.S., VAN MONTAGU M., INZÉ D., MAY M. J. & SUNG Z.R. 2000b The ROOT MERISTEMLESS1/CADMIUM SENSITIVE2 gene defines a glutathion-dependent pathway involved in initiation and maintenance during postembryonic root development. Plant Cell 12, 97-109.

VOLLBRECHT E., VEIT B., SINHA N. & HAKE S. 1991 The developmental gene *Knotted-1* is a member of a maize homeobox gene family. Nature 350, 241-243.

VROEMEN C.W., MORDHORST A.P., ALBRECHT C., KWAAITAAL M.A. & DE VRIES S.C. 2003 The CUP-SHAPED COTYLEDON 3 gene is required for boundary and shoot meristem formation in *Arabidopsis*. Plant Cell 15, 1563-77.

VUYLSTEKER C., DEWAELE E. & RAMBOUR S. 1998 Auxin in induced lateral root formation in chicory. Ann. Bot. 81, 449-454.

WADA T., KURATA T., TOMINAGA R., KOSHINO-KIMURA Y., TACHIBANA T., GOTO K., MARKS M. D., SHIMURA Y. & OKADA K. 2002 Role of a positive regulator of root hair development, CAPRICE, in *Arabidopsis* root epidermal cell differentiation. Development 129, 5409-5419.

WAITES R. & HUDSON A. 2001 The HANDLEBAR gene is required with PHANTASTICA to dorsoventral asymmetry of organs and for stem activity in *Antirrhinum*. Development 128, 1923-1931.

WAITES R., SELVADURAI H.R.N., OLIVER I.R. & HUDSON A. 1998 The PHANTASTICA gene encodes a MYB transcription factor involved in growth and dorsoventrality of lateral organs in *Antirrhinum*. Cell 93, 779-789.

WANG X., FENG S., NAKAYAMA N., CROSBY W. L., IRISH V., DENG X. W. & WEI N. 2003 The COP9 signalosome interacts with SCFUFO and participates in *Arabidopsis* flower development. Plant Cell 69, 1071-1082.

WANG G., KONG H., SUN Y., ZHANG X., ALTMAN N., DE-PAMPHILIS C.W. & MA H. 2004 Genome-wide analysis of the cyclin family in *Arabidopsis* and comparative phylogenetic analysis of plant cyclin-like proteins. Plant Physiol. 135, 1084-1099.

WANG H., QI Q.G., SCHORR P., CUTLER A.J., CROSBY W.L. & FOWKE L.C. 1998 ICK1, a cyclin dependent protein kinase inhibition from *Arabidopsis thaliana* interacts with both *Cdc2a* and *Cyc D3*, and its expression is induced by absisic acid. Plant J. 15, 501-510.

WEBB M.C. & GUNNING B.E.S. 1991 The microtubular cytoskeleton during development of the zygote, proembryo and free-nuclear endosperm in *Arabidopsis thaliana* (L.) Heynh. Planta 184, 187-195.

WEIGEL D. & MEYEROWITZ E. 1994 The ABCs of floral homeotic genes. Cell 78, 203-209.

WEIGEL D. & NILSSON O. 1995 A developmental switch sufficient for flower initiation in diverse plants. Nature 377, 495-500.

WEINL C., MARQUARDT S., KUIJT S.J.H., NOWACK M.K., JACOBY M.J., HULSKAMP M. & SCHNITTGER A. 2005 Novel functions of plant-dependent-kinase inhibitors ICK1/KRP1, can act non-cell-autonomously and inhibit entry into mitosis. Plant Cell 17, 1704-1722.

WERNER T., MOTYKA V., LAUCOU V., SMETS R., VAN ONCKELEN H. & SCHMÜLLING T. 2003 Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alteractions indicating opposite functions of cytokinins in the regulation of shoot and root meristems activity. Plant Cell 15, 2532-2550.

WHITE O.E. 1948 Fasciation. Bot. Rev. 14, 319-358.

WILLEMSEN V., WOLKENFELT H., DE VRIEZE G., WEISBEEK P. & SCHERES B. 1998 The HOBBIT gene is required for formation of the root meristem in the *Arabidopsis* embryo. Development 125, 521-531.

WILLIAMS R.W. 1998 Plant homeobox genes: many functions stem from a common motif. BioEssays 20, 280-282.

WOLFF C. F. 1759 *Theoria generationis.* Leipzig, Wilhelm Engelman

WYRZYKOWSKA J., PIEN S., SHEN W.H. & FLEMING A.J. 2002 Manipulation of leaf shape by modulation of cell division. Development. 129, 957-964.

ZAKRZEWSKI J. 1983 Hormonal control of cambial activity and vessel differentiation in *Quercus robur*. Physiol. Plant. 57, 537-542.

ZHANG K., LETHAM D.S. & JOHN P.C.L. 1996 Cytokinin controls the cell cycle at mitosis by stimulating the tyrosine dephosphorylation and activation of p34cdc2-like H1 histone kinase. Planta 200, 2-12.

Chapter 9
Somatic Embryogenesis

1. INTRODUCTION

During the course of evolution, many plant species have evolved different methods of asexual embryogenesis, including somatic embryogenesis, to overcome various environmental and genetic factors that prevent fertilization. Somatic embryogenesis is a process whereby somatic cells differentiate into somatic embryos. Somatic embryos resemble zygotic embryos morphologically. They are bipolar and bear typical embryonic organs. However, they develop via a different pathway. Somatic embryogenesis occurs to a limited extent under natural conditions, within ovules (e.g., *Paeonia*) and more rarely on leaves (e.g. *Asplenium* and *Kalanchoe*). Since the first observation of somatic embryo formation in *Daucus carota* cell suspensions by Steward *et al.* (1958) and Reinert (1958) the potential for somatic embryogenesis has been shown to be characteristic of a wide range of tissue culture systems in plants. During the past 40 years, somatic embryogenesis has been described in a large number of plant species. New species and modified methods are continuously reported and described and their number continuously increases. Methods for bringing about this kind of morphogenesis are also steadily being modified and improved. Somatic embryogenesis can probably be achieved for all plant species provided that the appropriate explant, culture media and environmental conditions are employed. In this chapter we shall highlight important aspects of somatic embryogenesis. We shall not give details for different species since this field of research is developing very fast and it is important to search for the latest information; this can easily be done on the web. Somatic embryos are used as a model system in embryological studies. However, the greatest interest of somatic embryos is centred in its practical application for large-scale vegetative propagation, particularly because of the possibility to scale up the propagation by using bioreactors (see Chapter 1). In addition, in most cases, somatic embryos or embryogenic cultures can be cryopreserved, which makes it possible to establish gene banks. Embryogenic cultures are also an attractive target for gene transformation.

2. PLANT EMBRYOGENESIS

Before starting to work with somatic embryogenesis, it is crucial to have a basic knowledge about the embryology of the species of interest. Plant embryogenesis begins with the zygote and passes through a stereotyped sequence of characteristic stages. Although considerable morphogenesis occurs after seed germination, the embryonic phase is crucial as it is here that meristems and the shoot-root body pattern are specified. Angiosperms and gymnosperms became separated about 300 million years ago. Since their embryology differs in many aspects we shall briefly describe embryology in one angiosperm, *Arabidopsis*, and one gymnosperm, *Pinus* (Fig. 9.1).

2.1. FERTILIZATION

One of the characteristics of angiosperms is the process of double fertilization, where both male gametes participate in an act of fusion: one unites with the egg cell to form the diploid zygote from which the embryo develops, while the other gamete fuses with the central cell of the embryo-sac which then develops into the triploid endosperm. The egg cell is polarized, having a nucleus at the cytoplasm-rich chalazal pole while the micropylar pole is highly vacuolated (Russell, 1993). The microtubular cytoskeleton and the location of actin microfilaments within the cytoplasm are also polarised (Dodeman *et al.*, 1997). The cytoplasm is predominantly maternally inherited in angiosperms, although there are species with uniparental, paternal or biparental cytoplasmic inheritance (Dumas *et al.*, 1998). Typically, an angiosperm zygote is briefly quiescent after karyogamy. The endosperm is first syncytial and later becomes cellular in most taxa (Otegui and Staehelin, 2000). The endosperm plays a role in embryo nutrition as it accumulates reserves of starch, proteins and lipids. Genetic analysis suggests that maternal and endosperm tissues may regulate each others' development (Lopes and Larkins, 1993).

Gymnosperms display single fertilization since only the larger male gamete migrates through the egg cytoplasm and fuses with the egg nucleus in the centre of the egg cell while the smaller male gamete degenerates. The zygote is surrounded by neocytoplasm consisting of a large fraction of the egg and

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male nucleoplasm, and some of the male cytoplasm (Singh, 1978). Typically, in gymnosperms, cytoplasmic inheritance has a biparental character with paternal inheritance of chloroplasts and maternal inheritance of mitochondria (Neale and Sederoff, 1989). The megagametophyte originates from the megaspore and undergoes development prior to fertilisation. The first phase of megagametophyte development is characterised by an extensive series of cell-free nuclear divisions. Then, wall formation begins at the periphery and proceeds towards the centre until the entire megagametophyte is cellular (Gifford and Foster, 1987). As the embryo begins to differentiate, the cells of the megagametophyte accumulate reserves of starch, proteins and lipids. In the early stages of development, the embryo is nourished by the egg cytoplasm through the suspensor and it is only later that it begins to draw upon the cells of the megagametophyte. However, the bulk of these reserves are held over by the megagametophyte for use by the germinating embryo (Raghavan and Sharma, 1995).

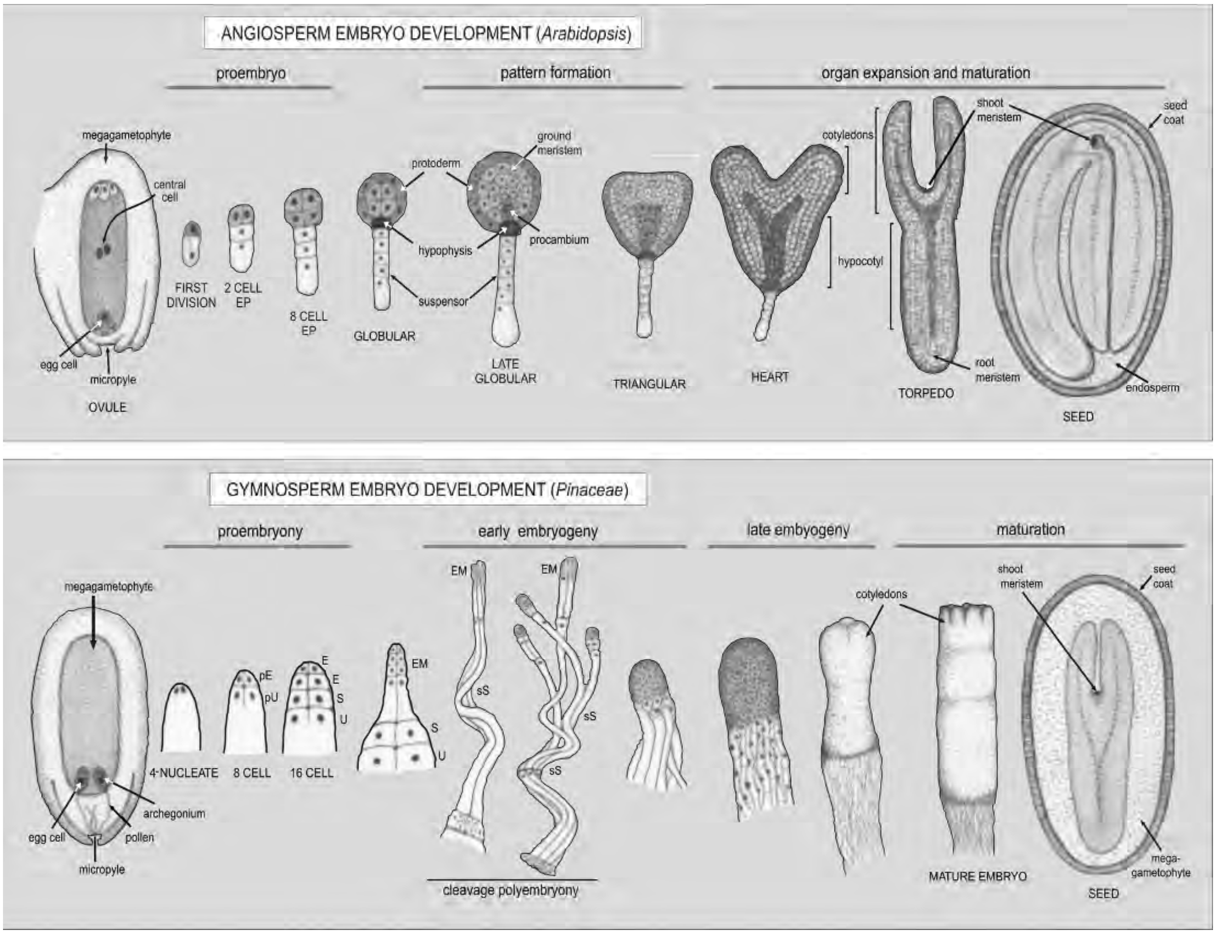

Fig. 9.1 Schematic overview of angiosperm *(Arabidopsis)* and gymnosperm *(Picea)* embryo development. The illustrations are not to scale. Drawings were prepared based on Goldberg *et al.* (1994) and Giffort and Foster (1987). Abbreviations: EP - embryo proper, pU - primary upper tier, pE – primary embryonal tier, E - embryonal tier, S - suspensor tier, U - upper tier, EM – embryonal mass, sS - secondary suspensor.

2.2. STAGES DURING EMBRYO DEVELOPMENT

Embryo development in angiosperms can be divided into two main steps (Dodeman *et al.,* 1997):

1. Embryogenesis *sensu stricto*, beginning with the zygote and finishing at the cotyledonary stage.

The development through the globular, heart, torpedo and cotyledonary stages can be divided into a sequence of different stages representing three major events (Goldberg *et al*., 1994):

i. Asymmetric division of the zygote, giving rise to a small apical cell and a large basal cell.
ii. Specific pattern formation, which takes place in the globular embryo.
iii. Transition to the cotyledonary stage which coincides with the initiation of the root primordium followed, in dicots, by the shoot primordium.

2. Maturation of the embryo followed by germination.

The sequence of embryo development in gymnosperms can be divided into three phases (Singh, 1978):

1. Proembryogeny - all stages before the elongation of the suspensor.
2. Early embryogeny - all stages after elongation of the suspensor and before establishment of the root meristem.
3. Late embryogeny - intensive histogenesis including establishment of the root and shoot meristems.

These three steps in gymnosperm embryo development correspond to the stages described above for embryogenesis *sensu stricto* in angiosperms and are followed by the period of embryo maturation.

2.3. ASYMMETRIC DIVISION OF THE ZYGOTE/PROEMBRYOGENY.

In angiosperms, the zygote divides a few hours after fertilisation at which time it already has a highly polarized appearance, displaying uneven distribution of organelles and metabolites (Goldberg *et al*., 1994). Such cytological and physiological polarity in the zygote affects the ultrastructure of the daughter cells. The first division is almost invariably asymmetric and transverse, cutting off a large vacuolated basal cell and a small, densely cytoplasmic terminal cell. The organogenic part of the embryo is derived from the terminal cell with little or no contribution from the basal cell. The asymmetric pattern of the first division is an important feature for the initiation of the embryogenic developmental pathway. It has been suggested that asymmetrical division is probably the consequence of nuclear migration from the central region of the cell to the periphery (Duditis *et al*., 1995).

There are four types of proembryogeny in gymnosperms, of which the conifer type is the most common and is interpreted as a basal plan for gymnosperm embryogeny (Wehmeyer *et al*., 1993). A common feature of gymnosperm embryo development is the free nuclear stage which, with the exception of the dicotyledonous genus *Paeonia* (Yakovlev and Yoffe, 1957), is not seen in any other plant group. The number of free nuclei differs among species. In *Picea* and *Pinus*, which represent the conifer type of embryogeny, four nuclei are present at the free nuclear stage. Proembryo development begins when the fertilised egg nucleus divides into two, then four, free nuclei contained within a dense region of cytoplasm (neocytoplasm). The four free nuclei are arranged in a tier at the chalazal end of the archegonial sac. After division, two tiers of four nuclei each are formed, the primary embryonal tier and the primary upper tier which soon become cellular. Internal divisions of these two tiers produce four tiers of four cells each. The lower two tiers constitute the embryonal tier, the tier above it is called the suspensor tier. Cells of this tier may elongate to form the primary suspensor; they are often dysfunctional but can show some meristematic activity. The uppermost tier degenerates. The upper four cells of the embryonal tier elongate to form a functional suspensor (called an embryonal suspensor) and the lower four cells of the embryonal tier form the embryonal mass. The proembryo stage lasts approximately one week.

2.4. PATTERN FORMATION IN THE GLOBULAR EMBRYO/EARLY EMBRYOGENY

In angiosperms the divisions of the zygote up to the globular stage embryo are in predictable planes (Raghavan and Sharma, 1995). When first formed, the embryo proper consists of a mass of relatively undifferentiated cells. Soon, however, changes in the internal structure of the embryo result in the initial development of the tissue system of the nascent plant. Two critical events take place after the embryo proper is formed (Goldberg *et al*., 1994):

1. the specification of the radial pattern with three primordial tissue layers, and
2. the differentiation of the regions along the apical-basal axis from which embryonic organ systems generate.

The first tissue to differentiate is the protoderm which is formed by periclinal divisions of cells of the early globular embryo. The formation of the protoderm, which restricts cell expansion, is essential for the remaining developmental phases (Dodeman *et al.,* 1997). Subsequent cell differentiation events within the embryo proper result in the production of an inner procambial layer and a middle layer of ground meristem cells. Differentiation of these three

tissue layers establishes a radial axis in the globular embryo (Goldberg *et al.,* 1994).

Three major regions can be distinguished along the axis of apical-basal polarity: an apical region giving rise to the shoot meristem and the cotyledons, a central region including hypocotyl, embryonic root (radicle) and the initials of the root cap and a basal region corresponding to the hypophysis, which gives rise to the quiescent centre of the root meristem and the central root cap (Jurgens *et al*., 1994).

During early embryogeny in gymnosperms, cells of the lower embryonal tier divide, creating an embryonal mass. The basal cells of the embryonal mass continue to divide predominantly in a transverse plane and elongate, contributing to the thick secondary suspensor. The lack of the restriction of cell divisions means that even the surface layers continue to divide periclinally as well as anticlinally, preventing differentiation of a distinct protoderm (Singh, 1978). However, this outer cell layer does function as a protoderm (Romberger *et al.,* 1993). The club-shape early embryo enlarges rapidly filling the corrosion cavity that forms in the female gametophyte (Owens and Molder, 1984).

2.5. ESTABLISHMENT OF ROOT AND SHOOT MERISTEMS/ATE EMBRYOGENY.

In dicotyledonous species, the root primordium emerges at the end of embryo pattern formation. Cotyledons are specified from two lateral domains at the apical end. The formation of cotyledons results in the characteristic heart stage. At the same time the hypocotyl region of the axis begins to elongate. Morphogenic changes during this period are mediated by differential cell division and expansion (Goldberg *et al*., 1994). The shoot meristem forms later in embryogenesis from cell layers localised in the upper axis between the two cotyledons. In monocotyledonous species both the root and the shoot meristems are laid down in a lateral fashion rather than distally. As a result, the axis of the mature embryo does not correspond to the axis of the proembryo (Mordhorst *et al*., 1997). The distal region above the shoot meristem greatly expands to form the scutellum. Late embryogeny in gymnosperms is a period of histogenesis and organogenesis. Early during this period, the root and shoot apical meristems are delineated and the plant axis is established. The root apical meristem forms near the centre of the embryo first as a root-organisation centre. The shoot meristem originates at the distal part of the globular embryonal mass and it is relatively superficial compared to root organisation centre. The cotyledon primordia arise in a ring around the distal end of the embryo. At this stage provascular tissue and cortex also differentiate. In *Pinaceae*, the protoderm covers only the shoot/hypocotyl region but in other gymnosperms it covers the entire surface of the embryo (Romberger *et al*., 1993).

2.6. MATURATION.

A remarkable change occurring during this period is that the developmental programme switches from pattern formation to storage product accumulation in order to prepare the young sporophyte for dormancy and postembryonic development. Following the previous period of cell division and histodifferentiation, there is a period of embryo development in which the major cell expansion and storage product deposition occur called the maturation phase. The maturation program is responsible for:

1. synthesising large amounts of storage products,
2. inducing water loss,
3. preventing premature germination, and
4. establishing a state of dormancy (Goldberg *et al.,* 1994).

The rate of synthesis and deposition of storage proteins, lipids and starch increases and results in cell expansion in both cotyledons and axis. Cell vacuoles exhibit a specialised behaviour during maturation in that they split up and dehydrate to give rise to protein bodies and aleurone grains (Dodeman *et al*., 1997; Bethke *et al*., 1999). At the end of the maturation phase seeds enter dormancy (Goldberg *et al*., 1989). An essential regulator of the process is ABA. The concentration of ABA peaks in abundance during late embryogenesis (Bewley and Black, 1994), modulating gene expression, at least at the transcriptional level. The mature seeds are classed as orthodox or recalcitrant (Engelmann, 1991). The embryos of orthodox seeds undergo maturation drying while recalcitrant seeds do not and are generally dessication intolerant. The majority of angiosperm and conifer seeds are of the orthodox type. Orthodox seeds can be further divided into quiescent or dormant type (Bewley and Black, 1994). Quiescent seeds can germinate after addition of water while dormant seeds require additional factors to germinate. Orthodox seeds are more resistant to diverse conditions and can survive in more extreme environments.

2.7. SUSPENSOR SYSTEMS

The suspensor is a dynamic temporal structure with important functions during embryogenesis (Yeung and Meinke, 1993). The suspensor functions early in embryogenesis and it then undergoes programmed cell death (Nagl, 1976; Meinke, 1995). It appears from extensive structural, biochemical, and physiological studies with a variety of angiosperms that the suspensor plays an active role early in development by promoting continued growth of the embryo proper. Being active nutrient transporters and important sources of plant growth regulators such as auxins, gibberellins, cytokinins and ABA (Raghavan and Sharma, 1995). during early stages of embryogenesis, suspensor cells often display increased transcriptional activity (Marsden and Meinke, 1985; Forino *et al.*, 1992; Yeung and Meinke, 1993). Compared to the cells of the embryo proper, suspensor cells contain more RNA and proteins and synthesise them more efficiently than embryo cells of the same age.

At the same time as the suspensor promotes growth of the embryo proper, suspensor growth is inhibited by the embryo proper (Mardsen and Meike, 1985; Yeung and Meinke, 1993). The developmental potential of the suspensor is often greater than its normal developmental fate. This potential is revealed only when the inhibitory effect of the embryo proper is disturbed, which leads to misregulation of programmed cell death in the suspensor, as evidenced from two *Arabidopsis* embryo specific mutants. One mutant (*twin*) displays abnormal proliferation of suspensors giving rise to multiple embryos (Vernon and Meinke, 1994; Zhang and Somerville, 1997), while another mutant *(raspberry*) fails to undergo the transition from globular to heart stage and continues to proliferate both in suspensor and embryo proper regions (Yadegari *et al.*, 1994).

However, somatic embryogenesis can sometimes occur without simultaneous development of a normal suspensor suggesting that either the suspensor does not play a crucial role in embryo development or components in the culture system replace the need for a suspensor. The non-embryogenic single cells present in embryogenic cultures of *Daucus carota* have been shown to have a stimulating effect on embryogenesis. Thus the suspension-cultured cells that have retained certain aspects of suspensor cells may possibly take over the role of the suspensor cells *in vitro*.

3. REGULATION OF EMBRYO DEVELOPMENT

During embryogenesis, the zygote undergoes a complex series of morphological and cellular changes that establish the morphogenetic pattern of the plant and the meristematic tissues required for post-embryonic development. Large numbers of genes must be expressed in a highly coordinated manner to ensure that the single-celled zygote develops into an organised, multicellular structure.

More than 3 x 10^4 different genes are expressed in embryos and seedlings (Thomas,1993). It was estimated that about 3500 different genes are necessary to complete embryo development. Of these, ca. 40 genes may direct the formation of all body pattern elements in the *Arabidopsis thaliana* embryo (Mayer *et al.*, 1991). However, progress in studying gene regulation during embryo development is limited by inaccessibility of the embryo, particularly at the early stages of embryogenesis. This has to some extent been overcome by the use of *in vitro* fertilisation, somatic embryogenesis and embryo defective mutants which have greatly increased our understanding of the regulation of embryo development.

3.1. ESTABLISHMENT OF CELL FATE IN THE EMBRYO

There are two mechanisms for generating cell fate diversity: unequal division of a polarized cell and position-dependent cell fate determination (Laux and Jurgens, 1997). Both these mechanisms operate during embryo development. The correct cell division planes play a crucial role during embryogenesis since division of a polarised cell partitions the cytoplasm and any regulatory molecules contained therein. As a result of asymmetrical divisions the daughter cells may inherit different cytoplasmic determinants and thereby acquire different fates. One transcription factor encoded by the *SHORT-ROOT* (*SHR*) gene, which is required for the asymmetric cell division responsible for formation of ground tissue (endodermis and cortex) as well as specification of endodermis in *Arabidopsis* roots, has been suggested to participate in a radial signalling pathway (Helariutta *et al.*, 2000).

The final cell fate is determined by positional information and/or by events established earlier on and transmitted down cell lineage. However, except for the early epidermal cell fate, no evidence has been

found for the existence of a rigid cell lineage in plants. Irish and Sussex (1992) suggested the term "probability map" rather than "fate map" to emphasise the absence of a rigid cell lineage. Cell position rather than previous developmental history is considered to be essential for the formation of somatic tissues (Dawe and Freeling, 1991). Therefore, in general terms, the establishment of cell fate during embryo development involves localised activity of specific regulatory proteins, as a consequence of the localised expression of particular genes (Lindsey and Topping, 1993). Although cell-cell communication is thought to play an important role in patterning of the plant embryo, the underlying molecular mechanisms is not understood. Two mechanisms have been proposed: signalling across the cell surface via leucine-rich repeat receptor serine-threonine kinases, and exchange of molecules through plasmadesmata (see review by Mayer & Jurgens, 1998).

Clonal analysis has indicated that stem cell fate is specified by positional information and imposed on the cells that currently reside at the summit of the shoot meristem. Only the progeny of the stem cells that stay at this position remain pluripotent, whereas daughter cells that leave this position differentiate (Mayer *et al*., 1998; Bowman and Eshed, 2000). Molecular mechanisms co-ordinating these two antagonistic processes in the *Arabidopsis* shoot meristem are now known to be controlled by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes (Schoof *et al.,* 2000).

3.2. EMBRYO MUTANTS

Mutagenesis and the subsequent identification and analysis of mutants have yielded several classes of mutant forms arrested at different stages of embryo development (Lotan *et al*., 1998, with refs.). Most screens have been done in *Arabidopsis*. Genetic analysis of different mutants leads to the conclusion that three basic elements of embryonic development, viz. pattern formation, morphogenesis and cytodifferentiation, are regulated independently.

Many of the mutants arrested early in development are likely to be affected in basic housekeeping functions that first become essential during early stages of development. Those which have been characterised are involved in basic functions such as biotin biosynthesis (*bio 1,* Shellhammer and Meinke, 1990), cell division and cell expansion (*EMB30*, Shevell *et al*., 1994) and intron splicing (*sus2*, Meinke, 1995). Other mutants are likely to be defective in genes that play a more direct role in plant growth and development. However, in some cases it may not be possible to make a clear distinction between housekeeping and regulatory functions because many genes are likely to perform cellular functions which are directly related to both growth and morphogenesis.

One class of mutations includes the suspensor mutants (*twin*, *raspberry*), which are an indirect consequence of altered balance between suspensor and embryo proper development (Vernon & Meinke, 1994; Yadegari *et al*., 1994; Zhang & Somerville, 1997). Disruption in signals between suspensor and embryo proper causes the suspensor to take on an embryo-like fate. Analysis of *raspberry* mutants, which are blocked at the globular stage but still differentiate tissue layers in their correct spatial context, indicates that tissue differentiation can take place independently of both organ formation and morphogenesis (Yadegari *et al*., 1994).

During embryo development three spatial domains are formed along the longitudinal axis: the apical domain composed of the cotyledons, shoot apex, and upper hypocotyl, the central domain including the majority of the hypocotyl and the basal domain consisting primarily of the root (West and Harada, 1993). Evidence for these domains was obtained by analysis of several apical-basal mutants, such as *gurke*, *fackel*, *monopteros* or *gnom* lacking one of the embryonal domains. Moreover, these domains can develop independently of each other (Goldberg *et al*., 1994). Although detailed mechanisms of this apical-basal axis formation are not yet clear, it has been postulated that they may originate from an intrinsic polarisation of the zygote, with the surrounding tissue possibly influencing the orientation of the axis (Mayer & Jurgens, 1998).

Analysis of *gnom* and *monopteros* mutants sheds light on the importance of auxins in pattern- and organ formation. The *MONOPTEROS* gene encodes a transcription factor, possibly involved in auxin signalling. A model was proposed in which a gradient distribution of auxins in the globular embryo is essential in mediating the transition from radial symmetry to bilateral symmetry, finally leading to shoot meristem formation (Fisher and Neuhaus, 1996). The GNOM protein was proposed to regulate vesicle trafficking required for the coordinated polar localisation of auxin efflux carriers which in turn determines the direction of auxin flow (Steinmann *et al*., 1999).

Nine recessive mutations derived from four independent loci causing the deletion of the shoot apical meristem (SAM), have been characterised in rice (Satoh *et al*., 1999; Chapter 8). Analysis of these mutants showed that differentiation of radicle and scutellum is regulated independently of SAM but that of coleoptile and epiblast may depend on SAM.

The formation of a SAM is the outcome of a successive patterning process initiated very early in embryo development. Once the SAM is established, expression of the *WUSCHEL* gene defines a group of cells that function to specify overlaying neighbours as stem cells (Mayer *et al*., 1998). The *CLAVATA* (*CLV1* and *CVL3*) and the *SHOOT MERISTEMLESS* (*STM*) genes specifically regulate shoot meristem development in *Arabidopsis*. *CLV* and *STM* have opposite functions: *clv1* and *clv3* mutants accumulate excess undifferentiated cells in the shoot meristem, while *stm* mutants fail to form the undifferentiated cells of the shoot meristem during embryonic development (Clark *et al*., 1996, with ref.). *CLV1,* which encodes a receptor kinase (Clark *et al*., 1997) acts together with *CLV3* to control the balance between meristem cell proliferation and differentiation (Fletcher *et al*., 1999).

Arabidopsis mutants with enlarged SAMs such as *clv* and *primordia timing* (*pt*) give rise to stable embryogenic cultures (Mordhorst *et al*., 1998). The authors suggest that an increased population of noncommitted SAM cells are responsible for facilitated establishment of somatic embryogenesis. Genetic studies have revealed that, in *Arabidopsis*, the *ABA-INSENSITIVE3* (*ABI3*), *FUSCA3* (*FUS3*) and *LEAFY COTYLEDON1* (*LEC1*) loci play major roles in regulating maturation (Kurup *et al*., 2000). All three promote embryo-specific processes and simultaneously repress germination. They interact to regulate several processes during seed maturation, including accumulation of chlorophyll, desiccation tolerance, sensitivity to ABA and expression of storage proteins (Parcy *et al.,* 1997; Wehmeyer & Vierling, 2000). *FUS3* and *LEC1* regulate abundance of the ABI1 protein. However, the *lec1* mutation is pleiotropic and by analysing the effects of the *lec1* mutation on embryo development it was shown that *LEC1* is an important regulator of embryo development that activates the transcription of genes required for both embryo morphogenesis and cellular differentiation (Lotan *et al*., 1998).

3.3. GENE EXPRESSION DURING EMBRYOGENESIS

Although the newly formed zygote contains both maternal and paternal genetic information, it seems that the activity of many genes acting during early embryo and endosperm formation may depend solely on transcription from the maternally inherited alleles since the paternal genome is initially silenced (Vielle-Calzada *et al*., 2000).

The general feature underlying the expression of embryo-specific mRNA sequences is that they appear and decay at various times during embryogenesis. This indicates that the expression of each set of genes is controlled by specific regulatory signals. What causes the activation of specific genes at specific stages of embryogenesis is not well understood.

The involvement of plant homeobox genes in embryogenesis was first demonstrated by the analysis of the *Arabidopsis stm* mutant. *STM* encodes a KNOTTED1 (KN1)- type homeodomain protein and is expressed in the region of SAM during embryogenesis (Smith *et al*., 1995). Localised expression of other *KN1*-type homeobox genes has also been observed around the region in which the SAM develops early in embryogenesis (Sentoku *et al*., 1999). Another example is the *ATML1* gene, belonging to the homeodomain-leucine zipper (*HD-Zip*) transcription factors, which is expressed specifically in the apical cell after the first division of the zygote. Later, at the globular stage, the expression of *ATML1* is restricted to the protoderm (Lu *et al*., 1996). Furthermore, a family of HD-Zip genes with cell-layer-specific expression patterns defining subdomains of the embryo and certain meristems have been isolated from maize (Ingram *et al*., 2000). An embryo-specific zinc finger protein gene, *PEI1*, plays an important role for the globular to heart-stage transition (Li and Thomas, 1998).

Characterisation of gene expression during embryo development, maturation and germination has led to the identification of distinct classes of developmentally regulated genes in angiosperms (Dure III 1985; Goldberg *et al*., 1989). These genes can be divided into five major classes as follows:

- *Class 1*. Constitutively expressed genes whose products are present at all stages and have functions required during normal plant growth. These genes have housekeeping functions essential for many plant cells, including those in the embryo.
- *Class 2*. Embryo-specific genes whose expression is restricted to the embryo proper, and ceases prior to or at germination.

- *Class 3*. Genes highly expressed during early embryogenesis until the cotyledonary stage.
- *Class 4*. Genes representing seed protein genes, expressed during the expansion of cotyledons and seed maturation.
- *Class 5*. Genes expressed abundantly in later stages of embryogenesis until seed maturation. The genes are activated by ABA.

Seed proteins, class 4, are encoded by several multigene families and are very abundant during the maturation stage of embryogenesis – representing up to 50% of the embryonal mRNAs at the midpoint of maturation. In the embryo the expression is localised within specific cells of the cotyledons and axis and is absent from the surrounding non-embryogenic seed tissues. Seed protein genes are regulated by both transcriptional and post-transcriptional processes (Goldberg *et al.*, 1989).

Among class 5 genes, late embryogenesis abundant (LEA) protein genes predominate (Dure III *et al.*, 1989; Thomas, 1993). LEA proteins share several common characteristics. They are all hydrophilic and contain a large number of uncharged and hydroxylated amino acids. They are thought to protect cellular membranes and proteins during desiccation and the subsequent period of dormancy. Since these genes can be induced in other parts of the plant by ABA or application of various stresses, their expression is not embryo-specific.

4. GENERAL ASPECTS OF SOMATIC EMBRYOGENESIS

Somatic embryos can either differentiate directly from the explant without any intervening callus phase or indirectly after a callus phase (Williams and Maheswaran, 1986). Explants from which direct embryogenesis is most likely to occur include microspores (microsporogenesis), ovules, embryos and seedlings.

A special case of direct somatic embryogenesis, is the process which usually is classified as secondary embryogenesis. Secondary embryogenesis is termed continuous, recurrent or accessory, when the first formed somatic embryo fails to develop into a plant but instead gives rise to successive cycles of embryos, secondary, tertiary etc. Secondary embryos develop directly from epidermal and subepidermal cells of the cotyledons or hypocotyls (Thomas *et al.*, 1976). In some cases the formation of secondary embryos is of significant importance for increasing the yield of plants regenerated. If directly formed somatic embryos are converted into plants, no further multiplication is possible. However, it is often difficult to stop the process and consequently no, or only a few, normal plants can be regenerated. The distinction between direct and indirect somatic embryogenesis is unclear except for secondary embryogenesis. The main problem is that according to older hypotheses direct embryogenesis should take place from embryogenic pre-determined cells. In contrast, indirect somatic embryogenesis should take place from undetermined cells and an undifferentiated callus should first be formed. However, in reality the callus formed is either embryogenic or not. It is usually easy to distinguish between embryogenic and non-embryogenic callus based on morphology and colour. Embryogenic callus is composed of proembryogenic masses (PEMs). At present it is not known if the first formed PEM actually is an embryo which deviates from the normal embryonic development in response to plant growth regulators (PGRs) and proliferates. Owing to the difficulty in strictly separating direct and indirect somatic embryogenesis, we will focus on what would be classified as indirect embryogenesis, where embryogenic callus is formed first (see Chapter 1). To execute this pathway efficiently, a number of critical physical and chemical treatments should be applied with proper timing. Although great progress has been made in the development of these treatments and understanding of their mechanism of action, it has also been revealed that some maturation treatments, coinciding with increased yield of somatic embryos, may cause adverse effects on embryo quality, thereby impairing germination and *ex vitro* growth of somatic embryo plants. Consequently, *ex vitro* growth of somatic embryo plants is under a cumulative influence of the treatments provided during the *in vitro* phase.

Plant regeneration via somatic embryogenesis includes five steps:

1. Intiation of embryogenic cultures by culturing the primary explant on medium supplemented with PGRs, mainly auxin but often also cytokinin.
2. Proliferation of embryogenic cultures on solidified or in liquid medium supplemented with PGRs, in a similar fashion to initiation.
3. Pre-maturation of somatic embryos in medium lacking PGRs; this inhibits proliferation and stimulates somatic embryo formation and early development.

4. Maturation of somatic embryos by culturing on medium supplemented with ABA and/or having reduced osmotic potential.
5. Regeneration of plants on medium lacking PGRs.

4.1. INITIATION OF EMBRYOGENIC CULTURES

Somatic cells within the plant contain all the genetic information necessary to create a complete and functional plant. The induction of somatic embryogenesis must then consist of the termination of a current gene expression pattern in the explant tissue and its replacement with an embryogenic gene expression programme. One possible mechanism for downregulation of current gene expression is DNA methylation, which is influenced by auxins (Lo Schiavo *et al*., 1989). It has also been proposed that PGRs and stress play a central role in mediating the signal transduction cascade leading to the reprogramming of gene expression. This results in a series of cell divisions that induce either unorganized callus growth or polarized growth leading to somatic embryogenesis (Dudits *et al*.,1995). The initiation of the embryogenic pathway is restricted only to certain responsive cells which have the potential to activate those genes involved in the generation of embryogenic cells. Only a few cells in the primary explant are competent for embryogenic induction, which according to Dudits *et al.* (1995) may be the result of varying auxin sensitivity of these cells. Alternatively it may be due to the presence of different auxin receptors, one responsible for cell division as such, and another responsible for the asymmetric division that generates an embryogenic cell (Lo Schiavo, 1994). Two mechanisms appear to be important for *in vitro* formation of embryogenic cells: asymmetric cell division and control of cell elongation (De Jong *et al.,* 1993; Emons, 1994). Asymmetric cell division is promoted by PGRs that alter cell polarity by interfering with the pH gradient or the electrical field around cells (Smith and Krikorian, 1990). The ability to control cell expansion is associated with polysaccharides of the cell wall and corresponding hydrolytic enzymes (De Jong *et al*., 1993; Emons, 1994; Fry, 1995).

Somatic embryogenesis has been described as being genetically determined. There are major genotype or cultivar differences for this trait. The choice of explant can in some species be of paramount importance for obtaining embryogenic cell-lines (Lo Schiavo, 1995, with references). Plant species which are capable of expressing their embryogenic potential regardless of the explant include *Daucus carota* and *Medicago sativa*, while for many other species embryonic or highly juvenile tissue has to be used as the explant. The pattern of the developmental response of cultured tissue is epigenetically determined and is influenced by the stage of development of the plant, the nature of the explant etc. (Litz and Gray, 1995).

Requirement for auxin or other PGRs for the initiation of somatic embryogenesis is largely determined by the developmental stage of the explant tissue. Usually an embryogenic callus is formed in the medium containing auxin. A nodular tissue is produced by the proliferation of PEMs, the process resembling a callus-like growth. Synthetic auxins are usually used for initiating embryogenic cultures. One mechanism whereby auxin may regulate embryogenesis, is through acidification of the cytoplasm and cell-wall (Kutschera, 1994). Most commonly used is 2,4-D (2,4-dichlorophenoxyacetic acid) at a concentration of one to ten micromolar. However, in some cases it is necessary to combine different auxins (see Chapter 5). Initiation of embryogenic cultures by cytokinin alone is relatively rare, but for many species it is important to combine auxin and cytokinin. In most species a low concentration of cytokinin (e.g. 0.1 to 1.0 μM) in the culture medium tends to stimulate the initiation of embryogenic cultures. At higher concentrations cytokinins may have a negative effect. Initiation can often be stimulated by either altering the concentration of exogenous applied auxins or the molar ratio of auxins to cytokinins. However, thidiazuron (TDZ) alone has been found to substitute for both auxin and cytokinin in many species (Murthy *et al*.,1998, with refs.). It was suggested that TDZ induces somatic embryogenesis by modulating the endogenous level of both auxin and cytokinin. In some cases a higher rate of somatic embryogenesis is obtained with TDZ than with other PGRs. Only in a few instances has ethylene been reported to stimulate somatic embryogenesis. For example, ethylene promoted somatic embryogenesis from leaf disks of *Coffea* cultured in the presence of isopentenyladenine as the sole exogenous PGR (Hatanaka *et al*., 1995).

More recently the application of the new generation of growth regulators such as oligosaccharides, jasmonate, polyamines and brassinosteroids (see Chapter 7) has proved to be useful for initiating somatic embryogenesis in many plant species.

In some species a remarkable stimulation of initiation of somatic embryogenesis has been

achieved with compounds other than PGRs. Reduced forms of nitrogen supplied to the medium are important; ammonium or amide nitrogen often stimulate somatic embryogenesis (see Chapter 4). The type of carbon source has also been reported to affect initiation in many species (see Chapter 4).

Wounded zygotic embryos of *Daucus carota* can initiate embryogenic cultures on hormone-free medium. The cultures can be maintained as unorganized embryogenic cell masses on hormone-free medium at pH 4.0, containing NH_4^+ as the sole nitrogen source (Smith and Krikorian, 1990).

4.2. PROLIFERATION OF EMBRYOGENIC CULTURES

Once embryogenic cells are formed, they continue to proliferate forming PEMs. Auxin is required for proliferation of PEMs but is inhibitory for the development of PEMs into somatic embryos (De Vries *et al*., 1988; Nomura and Komamine 1985; Filonova *et al*., 2000a). The degree of embryo differentiation which takes place in the presence of auxin varies in different species. It should be noted that depletion of auxin in the culture medium starts after only a few days. Consequently, if the cultures are not transferred to fresh medium each week, somatic embryos will start to develop.

Embryogenic callus is maintained in a medium similar to that used for initiation. The cultures can be maintained and increased on semi-solid medium. However, for large-scale propagation it is usually better to establish suspension cultures. In addition to the fact that proliferation rate is higher in suspension cultures, another advantage is that the cultures become more synchronized. In suspension cultures single cells and cell aggregates develop as separate structures. Thus, the cells can easily be separated by sieving or centrifugation and thereafter subcultured and manipulated as required. Low pH is essential for maintaining cultures in the proliferation phase. During each subculture period the pH of the medium drops, frequently from 5.8 to around 4. When preglobular stage PEMs of *Daucus carota* are subcultured on hormone-free medium buffered at pH 5.8 they promptly continue their development to globular, heart, torpedo and cotyledonary stage embryos (Smith and Krikorian, 1990). Embryogenic cultures of some species and some genotypes can be subcultured for a prolonged period on medium containing PGRs, and still retain their embryogenic potential i.e. the capacity to produce mature somatic embryos which can develop into plants. However, the risk of increasing the rate of somaclonal variation and diminishing the embryogenic potential accumulates with prolonged culture. In our laboratory we do not use embryogenic cultures which have been proliferating for more than six months. As soon as the embryogenic cell-lines have been established they are cryopreserved and successively thawed for use in different studies.

4.3. PRE-MATURATION OF SOMATIC EMBRYOS

Comprising a link between proliferation of PEMs and organized embryonic development, and at the same time separating these stages, PEM-to-embryo transition plays an important role. It seems likely that the inability of many embryogenic cell lines to form well-developed somatic embryos is to a great extent associated with disturbed or arrested PEM-to-embryo transition. Until the embryos have reached the appropriate developmental stage they should not be exposed to maturation treatments. Synthetic auxins, such as 2,4-D, which are particularly effective for promoting establishment and proliferation of embryogenic cultures, are usually not metabolized by the cells to the same extent as natural auxins. Hence, for stimulating further growth of the somatic embryos it is necessary to transfer the embryogenic cultures to medium lacking auxin. With the depletion of auxin the block on the expression of those genes required for the transition to the heart stage is removed (Zimmerman, 1993).

When cultures are transferred from proliferation medium to medium stimulating embryo development they consist of a mixture of single cells and cell aggregates. To synchronise the development, the cells can be washed and/or sieved.

4.4. MATURATION OF SOMATIC EMBRYOS

During the maturation stage somatic embryos undergo morphological and biochemical changes. The storage organs, the cotyledons, expand concomitant with the deposition of storage materials, the repression of germination and the acquisition of desiccation tolerance (Thomas, 1993). Somatic embryos accumulate storage products that exhibit the same characteristics as those of the zygotic embryos. The storage products are also targeted to the correct subcellular compartments (Merkele *et al*., 1995). However, the amount of a particular storage product, as well as the timing of its accumulation can differ between somatic and zygotic embryos (Merkele *et al*., 1995; Yeung, 1995). The synthesis and deposition of storage and late embryogenesis abundant (LEA) proteins during somatic and zygotic embryogenesis

are usually regulated through ABA- and water-stress-induced gene expression (Dodeman *et al.,* 1997).

In some species it is necessary to treat the embryogenic cultures with ABA, usually at concentrations of 10 to 50 μM, to stimulate maturation; for reasons unknown, this is especially the case for conifers. In other cases ABA is used for reducing the process of secondary embryogenesis or for inhibiting precocious germination. In general, treatment for one month is optimal. Prolonged treatment usually increases the number of mature embryos formed. However, long exposure to ABA can have a negative after-effect on plant growth (Bozhkov and von Arnold , 1998). Many other factors such as ethylene, osmotic stress, pH and photoperiod have been reported to influence somatic embryo maturation in different species.

Maturation of seed embryos is generally terminated by some degree of drying, which results in a gradual reduction of metabolism as water is lost from the seed tissue and the embryo passes into a metabolically inactive, or quiescent, state. The lapse into metabolic quiescence resulting from desiccation is important; in addition to conferring tolerance to extreme environmental stresses, it permits broad dispersal of seeds through time and space (Kermode, 1990). Hydration of the orthodox seed leads to its germination, which is an immediate response to a switch from the maturation programme to the germination programme. In contrast, recalcitrant embryos are unable to survive desiccation and do not cease development during maturation.

Somatic embryos of the recalcitrant-type seed do not naturally enter a period of developmental arrest. They germinate precociously but the resulting plants have a low survival rate. It is possible to induce quiescence in somatic embryos of orthodox seed type species by dehydration treatment. Numerous attempts to improve the quality of somatic embryos have shown the stimulatory role of low osmotic potential in the maturation medium in embryo development, both in angiosperms (McKersie and Brown, 1996) and gymnosperms (Attree and Fowke, 1993). The effect mimics water stress naturally imposed on orthodox seeds during later stages of maturation. This allows embryos to survive severe dehydration when moisture content can diminish to as low as 5% (Bewley and Black, 1994). Different osmotic agents, including low (e.g., inorganic salts, amino acids and sugars) and high molecular mass compounds [e.g., polyethylene glycols (PEG) and dextrans] can provide low osmotic potential medium. However, *in vitro* osmotic stress induced by PEG with a M_r >4000 most closely approaches water stress observed in cells of embryos and plants subjected to dehydration conditions (Rains, 1989). The reason is that larger molecules of PEG are not able to pass through the cell wall while water is withdrawn, leading to a reduced turgor pressure and more negative intracellular water potential. Although the addition of PEG to the maturation medium in many cases has been shown to stimulate maturation, there are also reports showing adverse effects of PEG on embryo germination and early post-germinative root growth (Bozhkov and von Arnold, 1998). In *Picea abies* we found that PEG-treatment exerted a deleterious effect on embryo morphology and root meristem development.

In some species, the maturation treatment is followed by partial desiccation which substantially enhances somatic embryo germination frequency. It has been suggested that the desiccation reduces endogenous ABA content (Kermode *et al.,* 1989) or changes the sensitivity to ABA (Finkelstein *et al.,* 1985). It is well known that the sensitivity of seeds to imbibitional stress is controlled by the initial moisture content of the seed and the rate at which water is taken up (e.g. Pollock, 1969). The interaction of these factors has a dramatic effect on germination and subsequent seedling vigour. Usually, a high rate of water uptake i.e. quick imbibition has a deleterious effect on germination (Vertucci, 1989). Therefore, it is important to keep the initial rate of water uptake at a low level. Different methods are used for desiccation (see for example Roberts *et al*., 1990). The easiest way is to place the mature embryos individually in empty petri dishes and keep the humidity of the surrounding air at such a level that the embryos are first dehydrated and then hydrated at an appropriate rate.

4.5. REGENERATION OF PLANTS

Somatic embryogenesis is a complex process where the quality of the final product, i.e. survival and growth of regenerated plants, depends on the conditions provided at earlier stages, when mature somatic embryos are formed and germinate. Therefore, in order to develop mass propagation of somatic embryo plants, a dissection of critical factors that might contribute to *ex vitro* performance of plants is required. Only those mature embryos which have accumulated enough storage materials and acquired desiccation tolerance at the end of maturation develop into normal plants. Somatic

embryos usually develop into small plants, comparable to seedlings, on culture medium lacking PGRs. However, there are cases when auxin and cytokinin stimulate germination. Furthermore, a marked alteration in basal medium is often required. For some species, inclusion of extra compounds, like glutamine and casein hydrolysate, is also required. When the plants have reached a suitable size they can be transferred *ex vitro*. In many reports it has been shown that somatic embryo plants grow in a similar way to plants derived from true seeds. However, for some species somaclonal variation is a problem. In general, the use of 2,4-D and/or a prolonged callus phase are responsible for inducing genetic as well as epigenetic variation.

5. CONDITIONING FACTORS REGULATING SOMATIC EMBRYOGENESIS

The mechanism that controls cell differentiation in plant somatic embryos is far from clear, although there is evidence that soluble signal molecules are involved. It has long been observed that conditioned medium from embryogenic cultures could promote embryogenesis. For example, conditioned growth medium from highly embryogenic cultures can induce embryogenesis in non-embryogenic cultures (Hari, 1980). Growth medium preconditioned by a high-density suspension culture can also induce embryogenesis in cells cultured at a low cell density (Smith and Sung, 1985; de Vries *et al.*, 1988). This ability of conditioned medium to sustain or stimulate somatic embryogenesis implies that secreted soluble signal molecules are important.

5.1. EXTRACELLULAR PROTEINS

Secretion of proteins into the growth medium of suspension cultures has been reported in several species. However, only a few reports show that some specific secreted proteins can influence the development of somatic embryos. One of the proteins that promoted somatic embryo development in embryogenic cultures of *Daucus carota* was identified as a glycosylated acidic endochitinase (de Jong *et al.*, 1992). The authors studied the temperature sensitive variant *ts11* where somatic embryogenesis at the non-permissive temperature is blocked at the globular stage. Addition of the endochitinase to *ts11* embryogenic cultures rescued embryogenesis and promoted embryo formation. In similar experiments, an endochitinase from sugar beet stimulated early development of somatic embryos in *Picea abies* (Egertsdotter and von Arnold, 1998). In embryogenic cultures of *Daucus carota*, expression of an endochitinase gene was related to a certain population of cells that might have a nursing role in embryogenesis (van Hengel *et al.*, 1998). The exact role of the chitinases in somatic embryogenesis is not clear, but it has been suggested that chitinases are involved in the cleaving off of signalling molecule(s) from an as yet unknown substrate.

5.2. ARABINOGALACTAN PROTEINS

Arabinogalactan proteins (AGPs) have also been found to be important for the development of somatic embryos. AGPs are a heterogenous group of structurally complex macromolecules composed of a polypeptide, a large branched glycan chain and a lipid (Majewska-Sawka and Nothnagel, 2000). They are distinguished by their high carbohydrate-to-protein ratio, often more then 90% carbohydrate. AGPs are present in cell walls and plasma membranes, and commonly found in culture media. Perturbation of AGPs was found to alter somatic embryogenesis, indicating the importance of AGPs for embryo development. For example, addition of Yariv reagent, a synthetic phenyl-glycoside that specifically binds AGPs, to the culture medium blocked somatic embryogenesis in *Daucus* and *Cichorium* (Thompson and Knox, 1998; Chapman *et al.*, 2000). Precipitating AGP molecules with an anti-AGP antibody had a similar inhibitory effect on somatic embryo formation (Butowt *et al.*, 1999). Furthermore, addition of AGPs to the cultures was found to promote somatic embryogenesis. AGPs isolated from *Daucus* seeds restored the embryogenic potential in old cell lines of *Daucus* that had lost the ability to develop somatic embryos (Kreuger and van Holst 1993). Similarly, AGPs isolated from seeds of *Picea* promoted formation of more developed somatic embryos in low embryogenic cell lines of *Picea* (Egertsdotter and von Arnold, 1995). AGPs isolated from seeds of *Daucus* were fractionated using their binding to the antibodies ZUM15 and 18 (Kreuger and van Holst 1995). The ZUM15-reactive AGPs were inhibitory for somatic embryogenesis, while the ZUM18 reactive AGPs drastically increased the percentage of embryogenic cells and subsequently the number of embryos. Furthermore, ZUM18-reactive AGPs from *Daucus* enhanced the frequency of somatic embryos in *Cyclamen* (Kreuger *et al.*, 1995), and AGPs from *Lycopersicum* promoted somatic embryogenesis in *Daucus* (Kreuger and van Holst 1996). JIM4-reactive epitopes of AGPs were

found in embryogenic cells of *Daucus carota* and *Zea mays* (Kreuger and van Holst, 1996; Samaj *et al.*, 1999). On the other hand, JIM8-reactive AGP-epitopes were found in a subpopulation of *Daucus carota* cells with specific "nursing" function during somatic embryogenesis (McCabe *et al.*, 1997). Using JIM8 antibodies, McCabe *et al.* (1997) found two types of cells in embryogenic cultures of *Daucus carota*, - those that have JIM8-reactive AGP-epitopes (JIM8-positive cells) and cells devoid of JIM8-reactive epitopes (JIM8-negative cells). Further studies revealed that JIM8-negative cells in an unfractionated suspension develop into somatic embryos. At the same time, an isolated subpopulation of JIM8-negative cells could not form embryos. However, isolated JIM8-negative cells developed into embryos when supplemented with media conditioned by JIM8-positive cells. The data indicate that an active compound is released from JIM8-positive cells into the medium. Analysis of the chemical structure of this compound revealed the presence of both carbohydrate and lipid parts (McCabe *et al.*, 1997). It has been suggested that in embryogenic cultures of *Daucus carota* AGPs give rise to an oligosaccharin(s) with signalling functions (Darvill *et al.*, 1992). The presence of a glycosyl-phosphatidylinositol lipid anchor on some AGPs supports the possibility that AGPs are putative precursors of signalling molecule(s) (Svetek *et al.*, 1999). Although the chemical structure of the oligosaccharin(s) remains obscure, an increasing amount of data implicates lipochitooligosaccharides (LCOs) as signalling molecules in somatic embryogenesis.

5.3. LIPOCHITOOLIGOSACCHARIDES

Lipochitooligosaccharides (LCOs) are a class of signalling molecules that promote division of plant cells. It has long been known that LCO signals, Nod factors, secreted by *Rhizobium*, induce cell divisions in the root cortex, leading to the formation of nodules that can then be colonized (Spaink *et al.,* 1991, Truchet *et al.,* 1991). Nod factors produced by different *Rhizobium* species uniformly consist of an oligosaccharide backbone of 1,4-linked N-acetyl-D-glucosamine (GlcNAc) residues varying in length between 3 and 5 sugar units, and always carry an N-acyl chain at the non-reducing terminus. This basic structure is essential for the infection leading to nitrogen-fixing nodules. At the same time, several lines of evidence suggest the involvement of LCOs in regulating somatic embryo development. Rhizobial Nod factors were found to stimulate somatic embryos of *Daucus carota* to proceed to the late globular stage (De Jong *et al.*, 1993) and promoted the development of larger PEMs from small cell aggregates and further somatic embryo development in *Picea abies* (Egertsdotter and von Arnold, 1998; Dyachok *et al.*, 2000). More detailed studies showed that Nod factors could substitute for auxin and cytokinin to promote cell division. Furthermore, the Nod factor-like endogenous LCO compound(s) have been found in the conditioned medium from embryogenic cultures of *Picea abies*. A partially purified fraction of LCOs stimulated formation of PEMs and somatic embryos in *Picea abies* (Dyachok *et al.,* 2002). Remarkably, both in *Daucus carota* and *Picea abies* embryogenic systems, rhizobial Nod factors could substitute for chitinases in their effect on early somatic embryo development (De Jong *et al.*, 1993; Egertsdotter and von Arnold, 1998), indicating convergence of the activated signalling pathways. Evidence for concerted action in somatic embryogenesis of AGPs, chitinases and GlcNAc-containing oligosaccharides has been obtained. GlcNAc-containing AGPs were isolated from immature seeds of *Daucus carota*. The addition of AGPs increased the number of somatic embryos in embryogenic cultures of *Daucus carota*, and preincubation of the seed AGPs with endochitinases further increased the frequency of embryo formation (van Hengel, 1998). Taken together, the data support a hypothesis that endogenous LCOs analogous in structure to rhizobial Nod factors are released from AGPs by the action of endochitinases and act as signal molecules stimulating the development of somatic embryos.

6. TRACKING OF SOMATIC EMBRYOGENESIS

In order to efficiently regulate the formation of plants via somatic embryogenesis it is important to understand how the somatic embryo develops. Ideally, such knowledge should be gained through the construction of a fate map representing an adequate number of morphological and molecular markers specifying distinct developmental stages within the whole process (Irish and Sussex, 1992, Strehlow and Gilbert, 1993). When constructed, the fate map showing the correct progression of somatic

embryogenesis would provide the basis for further analyses of specification, induction and patterning of the embryonic tissues and organs.

6.1. CONSTRUCTION OF FATE MAPS

Construction of a fate map in somatic embryogenesis can be based on two alternative approaches either using synchronous cell-division systems (Nomura and Komamine, 1985; Tsukahara and Komamine, 1997; Osuga *et al*., 1999), or time-lapse tracking of the development of individual protoplasts, cells and multicellular structures (Golds *et al.,* 1992; Toonen *et al.,* 1994; Filonova *et al*., 2000a; Somleva *et al*., 2000). The second approach usually yields more consistent data, because there is no need to use cell-cycle-affecting drugs and centrifugation treatments which otherwise might interfere with embryonic development. Another potential advantage of the time-lapse tracking technique is that the starting individual cell, or cell aggregate, can be pre-selected based on certain criteria, and that it is possible to perform simultaneous analysis of dynamics and distribution of injected molecular probes conjugated with low molecular weight fluorochromes or fused to green fluorescent protein.

6.2. ANGIOSPERMS

In angiosperms, *Daucus carota* is the best understood species in terms of developmental pathways of somatic embryogenesis and their molecular mechanisms. This fact is not altogether surprising since *Daucus carota* embryogenic cell suspensions are highly amenable to both synchronization treatments and time-lapse tracking analysis (Zimmerman, 1993; Toonen and de Vries, 1997). By using the latter approach, Toonen *et al.* (1994) have studied three important aspects of somatic embryogenesis:

1. cell types which possess embryogenic potential, (i.e. able to develop into somatic embryos),
2. major stages of somatic embryogenesis in common for different initial cell types, and
3. variations and similarities between the pathways of somatic embryogenesis initiated from different cell types.

Based on shape (spherical, oval or elongated) and the density of cytoplasm (i.e. distinction between cytoplasm-rich and vacuolated cells), the authors have classified single cells isolated from embryogenic *Daucus carota* suspensions into five types, and found that cells of all types can develop into somatic embryos, albeit with a different frequency (varying between 19 and 100 somatic embryos per 10,000 cells). Embryo formation, in all cases, proceeded through the same stereotyped sequence of stages, i.e., state-0, and 2 cell clusters, according to Komamine *et al.* (1990). Auxin is required for the formation of a state-0 cell cluster, while subsequent stages, state-1 and 2 cell clusters, occur following withdrawal of auxin. Interestingly, depending on initial cell type, embryogenesis could occur via three different pathways distinguished by a lack or presence of geometrical symmetry of cell clusters at all three above-mentioned developmental stages. Oval and elongated cells, which were always vacuolated, developed into somatic embryos via asymmetrical cell clusters. Spherical cells which were either vacuolated or cytoplasm-rich, developed via symmetrical cell clusters into somatic embryos. Cells of a more variable initial morphology first developed aberrantly shaped cell clusters which then transformed into somatic embryos (Toonen *et al*., 1994). These observations imply that organised growth and polarity may not always occur during *in vitro* somatic embryogenesis, suggesting that in somatic embryogenesis, auxin-induced PEMs may be intermediates between unorganised growth and conservative embryonic pattern formation.

6.3. GYMNOSPERMS

A time-lapse tracking technique has been used to analyse developmental pathway of somatic embryogenesis in *Picea abies* (Filonova *et al*., 2000). A representative example of how *Picea abies* somatic embryos form and develop is shown in Fig. 9.2. It is noteworthy that, in contrast to *Daucus carota*, when a single cell fraction was obtained through fractionation of crude suspensions of *Picea abies*, neither densely cytoplasmic nor vacuolated cells on their own could develop into somatic embryos. Therefore the tracking began with a few-celled aggregates, called PEM I-cell aggregates. They are composed of a small compact clump of densely cytoplasmic cells adjacent to a single vacuolated cell. When immobilised in a thin layer of agarose, under appropriate trophic conditions and an initial supply of auxin and cytokinin, a PEM I-cell aggregate formed an additional vacuolated cell, thereby progressing to the next stage, PEM II (day 3; Fig. 9.2). Evans blue staining intensity of the primary vacuolated elongated cell increased by day 3 and was higher compared to newly formed additional vacuolated cells. In one of the vacuolated cells within PEM II it was possible to detect, by using the terminal deoxynucleotidyl

transferase-mediated dUTP nick end labelling (TUNEL) assay, the first signs of nuclear DNA degradation during the somatic embryogenesis pathway (Filonova *et al*., 2000b). Successively, PEM II enlarged in size by producing more cells of both types, while maintaining a bipolar pattern (days 5-10; Fig. 9.2.). The polarity and the distinct centre of densely cytoplasmic cells were lost after day 10 owing to the increased proliferative activity of densely cytoplasmic cells. This process continued from day 15 onward leading to formation of PEM III (days 15, 20; Fig. 9.2), followed by transdifferentiation of somatic embryos from PEM III (day 25; Fig. 9.2). Coincidentally, with somatic embryo formation, the primary structure, viz. PEM III, degenerates through massive PCD. Withdrawal of plant growth regulators (PGRs) dramatically activates both embryo formation and PCD in PEMs (Filonova *et al*., 2000b). In cell tracking analysis, embryos started to develop in PGR-free medium, which supported embryonic development through almost the entire period of early embryogeny (day 25-45, Fig. 9.2). For further embryo development and maturation, agarose layers with early somatic embryos must be replenished with ABA-containing medium. The first visible response of early somatic embryos to ABA is that the embryonal mass became opaque (day 4 ABA; Fig. 9.2). This response was accompanied by the elimination of the suspensor through PCD (Filonova *et al*., 2000b). Thereafter embryos started to elongate (days 7-14 ABA; Fig. 9.2) and differentiate cotyledons (days 20-35 ABA; Fig. 9.2). The tracking was terminated when fully matured somatic embryos resembling their zygotic counterparts had formed (day 45; Fig. 9.2).

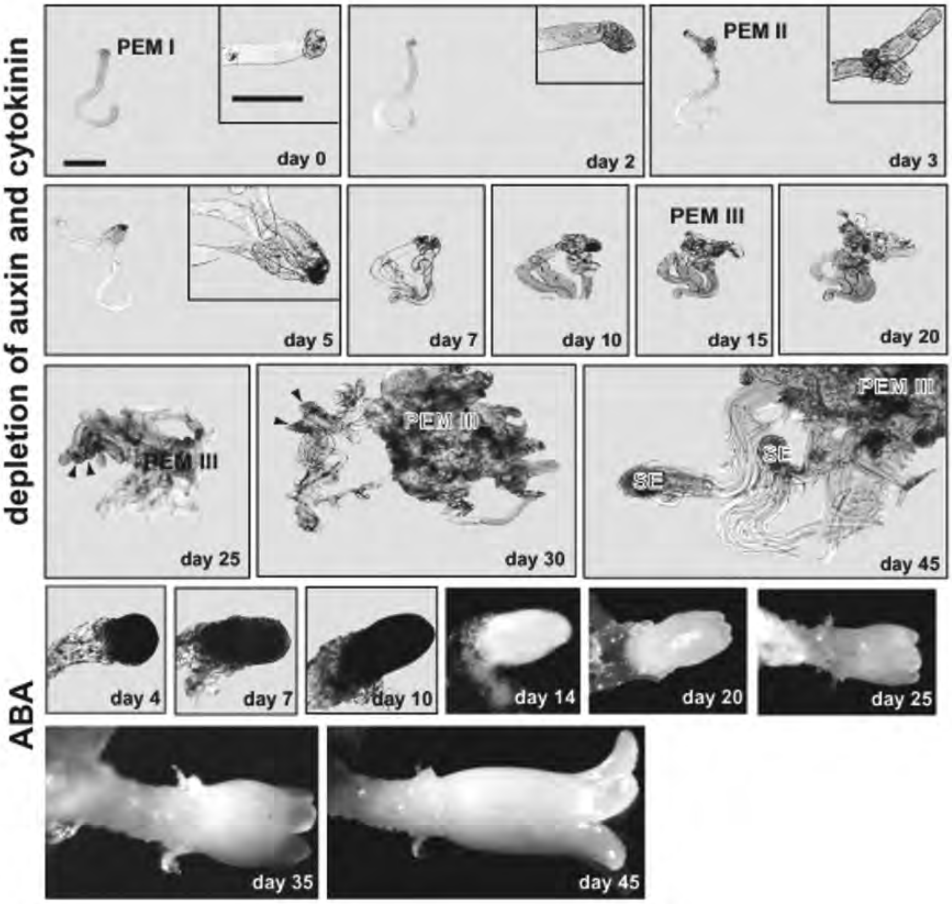

Fig. 9.2 Time-lapse tracking of somatic embryo formation and development in Norway spruce. The tracking was performed in a thin layer of agarose, first under gradual depletion of auxin and cytokinin and then following addition of ABA (for details see Filonova *et al.,* 2000a). Arrowheads denote somatic embryos (SE) transdifferentiating from PEM III. Bars = 250 µm.

6.4. MODEL FOR SOMATIC EMBRYOGENESIS

Based on the knowledge gained from studying the developmental pathway of somatic embryogenesis it is possible to construct a model of the process. The model has to be specific for each system. Below we give an example of a model for somatic embryogenesis in Norway spruce. Schematically shown in Fig. 9.3, somatic embryogenesis in *Picea abies* involves two broad phases, which in turn are divided into more specific developmental stages. The first phase is represented by proliferating PEMs,– cell aggregates which can pass through a series of three characteristic stages distinguished by cellular organization and cell number (stages PEM I, II and III), but can never develop directly into a real embryo. The second phase encompasses development of somatic embryos. The latter arise *de novo* from PEM III, and then proceed through the same, stereotyped sequence of stages as described for zygotic embryogeny of Pinaceae (Singh, 1978). Auxins and cytokinins are necessary during the first phase to maintain PEM proliferation, whereas embryo formation from PEM III is triggered by the withdrawal of PGRs. Once early somatic embryos have formed, their further development to mature forms requires ABA (Fig. 9.3). Each developmental stage within this fate map should be further characterised by a set of adequate molecular markers. What is emerging from our recent studies is that certain epitopes of arabinogalactan proteins and a lipid transfer protein are candidate markers (Dyachok *et al*., 2000; Filonova *et al*., 2000a, Sabala *et al*., 2000). Furthermore, a number of recently-described cytological and biochemical hallmarks of cell dismantling recruited during two waves of developmental PCD, viz. responsible for degradation of PEMs and elimination of suspensor (Filonova *et al*., 2000), will be an important adjunct to the fate map of *Picea abies* somatic embryogenesis.

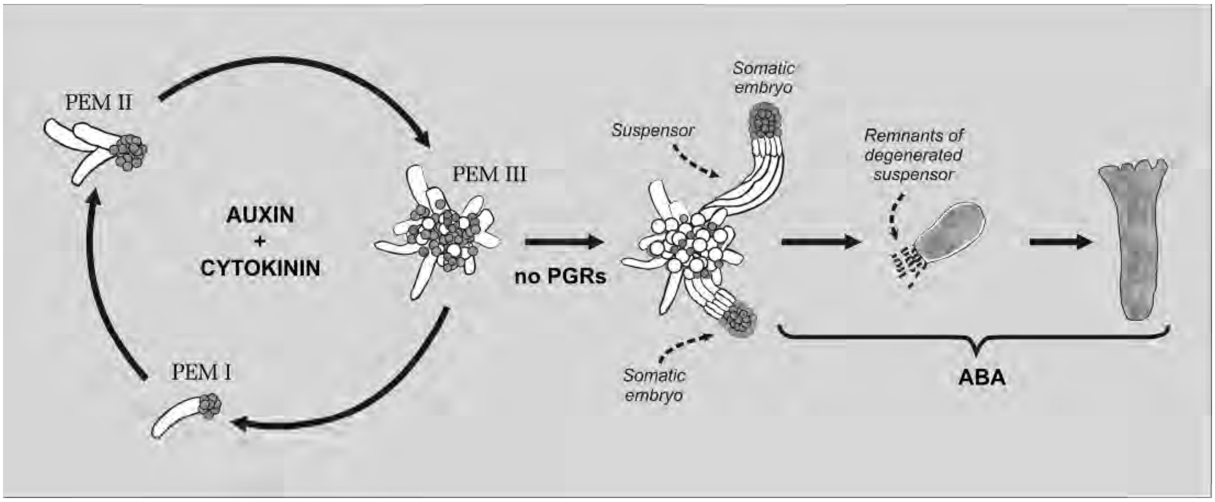

Fig. 9.3 A schematic representation of the developmental pathway of somatic embryogenesis in Norway spruce. Shown as dashed lines in the last but one stage of the pathway are the remnants of the degenerated suspensor in the beginning of late embryogeny.

REFERENCES

ATTREE S.M. & FOWKE L.C. 1993 Embryogeny of gymnosperms: Advances in synthetic seed technology of conifers. Plant Cell Tissue Organ Cult. 35, 1-35

BETHKE P.C., LONSDALE J.E., FATH A. & JONES R.L. 1999 Hormonally regulated programmed cell death in barley aleurone cells. Plant Cell 11, 1033-1045.

BEWLEY J.D. & BLACK M. 1994 *Seeds: Physiology of development and germination*. Plenum Press, New York.

BOWMAN J.L. & ESHED Y. 2000 Formation and maintenance of the shoot apical meristem. Trends Plant Sci. 3, 110-115.

BOZHKOV P. & VON ARNOLD S. 1998 Polyethylene glycol promotes maturation but inhibits further development of *Picea abies* somatic embryos. Physiol. Plant. 104, 211-224.

BUTOWT R., NIKLAS A., RODRIGUES-GARCIA M.I. & MAJEWSKASAWKA A. 1999 Involvement of JIM13 and JIM8-responsive carbohydrate epitopes in early stages of cell wall formation. J. Plant Res. 112, 107-116

CHAPMAN A., BLERVACQ A.-S., VASSEUR J. & HILBERT J.-L. 2000 Arabinogalactan proteins in *Cichorium* somatic embryogenesis: effect of (–glucosyl Yariv reagent and epitope localisation during embryo development. Planta 211, 305-314.

CLARK S.E., JACOBSEN, S.E., LEVIN J.Z. & MEYEROWITZ E.M. 1996 The CLAVATA and SHOOT MERISTEMLESS loci competitively regulate meristem activity in *Arabidopsis*. Development 122, 1567-1575.

CLARK S.E., WILLIAMS R.W. & MEYEROWITZ E.M. 1997 The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. Cell 89, 575-585.

DARVILL A., AUGUR C., BERGMANN C., CARLSON R.W., CHEONG J.-J., EBERHARD S., HAHN M.G., LO V.-M., MARFA V., MEYER B., MOHNEN D., O'NEILL M.A., SPIRO M.D., VAN HALBEEK H., YORK W.S. & ALBERSHEIM P. 1992 Oligosaccharins – oligosaccharides that regulate growth, development and defence responses in plants. Glycobiology 2, 181-198.

DAWE R.K. & FREELING M. 1991 Cell lineage and its consequences in higher plants. Plant J. 1, 3-8.

DE JONG A.J., SCHMIDT E.D.L. & DE VRIES S.C. 1993 Early events in higher plant embryogenesis. Plant Mol. Biol. 22, 367-377.

DE JONG A.J., CORDEWENER J., LO SHIAVO F., TERZI M., VANDEKERCKHOVE J., VAN KAMMEN A. & DE VRIES S.C. 1992 A *Daucus carota* somatic embryo mutant is rescued by chitinase. Plant Cell 4, 425-433.

DE JONG A.J., HEIDSTRA R., SPAINK H.P., HARTOG M.V., MEIJER E.A., HENDRIKS T., LO SHIAVO F., TERZI M., BISSELING T., VAN KAMMEN A. & DE VRIES S.C. 1993 *Rhizobium* lipo-oligosacharides rescue a *Daucus carota* somatic embryo variant. Plant Cell 5, 615-620.

DE VRIES S.C., BOOIJ H., MEYERINK P., HUISMAN G., WILDE D.H., THOMAS T.L. & VAN KAMMEN A. 1988 Acquisition of embryogenic potential in carrot cell-suspension culture. Planta 176, 196-204.

DODEMAN V.L., DUCREUX G. & KREIS M. 1997 Zygotic embryogenesis versus somatic embryogenesis. J. Exp. Bot. 48, 1493-1509.

DUDITIS D., GYORGYEYJ., BOGRE L. & BAKO L. 1995 Molecular biology of somatic emryogenesis. pp. 267-308 in Thorpe T.A. (ed.) *In vitro embryogenesis in plants*. Kluwer Academic Publishers, Dordrecht, Boston, London.

DUMAS C., BERGER F., FAURE J.-M. & MATTHYS-ROCHON E. 1998 Gametes, fertilization and early embryogenesis in flowering plants. Adv. Bot. Res. 28, 231-261.

DURE III L. 1985 Embryogenesis and gene expression during seed formation. Oxford Surv. Plant Mol. Cell Biol. 2, 179-197.

DURE III L., CROUCHE M., HARADA J., HO T.-H.D., MUNDAY J., QUATRANO R., THOMAS T., ANDE SUNG Z.R. 1989 Common amino acid sequence domains among the LEA proteins of higher plants. Plant Mol. Biol. 12, 475-486.

DYACHOK J.V., TOBIN A.E., PRICE N.P.J. & VON ARNOLD S. 2000 Rhizobial Nod factors stimulate somatic embryo development in *Picea abies*. Plant Cell Rep. 19, 290-297.

DYACHOK J.V., WIWEGER M., KENNE L. & VON ARNOLD S. 2002 Endogenous Nod-factor-like signal molecules promote early somatic embryo development in Norway spruce. Plant Physiol. 128, 523-533.

EGERTSDOTTER E.-M.U. & VON ARNOLD S. 1995 Importance of arabinogalactan proteins for the development of somatic embryos of Norway spruce (*Picea abies*). Physiol. Plant. 93, 334-345.

EGERTSDOTTER E.-M.U. & VON ARNOLD S. 1998 Development of somatic embryos in Norway spruce. J. Exp. Bot. 49, 155-162.

EMONS A.M.C. 1994 Somatic embryogenesis: cell biological aspects. Acta Bot. Neerl. 43, 1-14.

ENGELMANN F. 1991 *In vitro* conservation of tropical plant germplasm: a review. Euphytica 57, 227-243.

FILONOVA L., BOZHKOV P. & VON ARNOLD S. 2000a Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking. J. Exp. Bot. 51, 249-264.

FILONOVA L., BOZHKOV P., BRUKHIN, V., DANIEL G., ZHIVOVSKY B. & VON ARNOLD S. 2000b Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, Norway spruce. J. Cell Sci. 113, 4399-4411.

FINKELSTEIN R.R., TENBARGE K.M. & SHUMWAY J.E. 1985 Role of ABA in maturation of rapeseed embryos. Plant Physiol. 78, 630-636.

FISHER C. & NEUHAUS G. 1996 Influence of auxin on the establishment of bilateral symmetry in monocots. Plant J. 5, 621-630.

FLETCHER J.C., BRAND U., RUNNING M.P., SIMON R. & MEYEROWITZ E.M. 1999 Signaling of cell fate decisions by CLAVATA3 in *Arabidopsis* shoot meristems. Science 283, 1911-1914.

FORINO L.M.C., TAGLIASACCHI A.M., CAVALLINI A., CIONINI G., GIRALDI E. & CIONINI P.G. 1992 RNA-synthesis in the embryo suspensor of *Phaseolus coccineus* at 2 stages of embryogenesis, and the effect of gibberellic-acid. Protoplasma 167, 152-158.

FRY S.C. 1995 Polysaccharide-modifying enzymes in the plant-cell wall. Annu. Rev. Plant Physiol. Mol. Biol. 46, 497-520.

GIFFORD E.M. & FOSTER A.S. 1987 *Morphology and evolution of vascular plants.* W.H. Freeman & Co., New York.

GOLDBERG R.B., BARKER S.J. & PEREZ-GRAU L. 1989 Regulation of gene expression during plant embryogenesis. Cell 56, 149-160.

GOLDBERG R.B., DEPAIVA G. & YADEGARI R. 1994 Plant embryogenesis: zygote to seed. Science 266, 605-614.

GOLDS T.J., BABCIZINSKY J., RAUSCHER G. & KOOP H.-U. 1992 Computer-controlled tracking of single cell development in *Nicotiana tabacum* L. and *Hordeum vulgare* L. protoplasts embedded in agarose/alginate films. J. Plant Physiol. 140, 582-587.

HATANAKA T., SAWABE E. & AZUMA T. 1995 The role of ethylene in somatic embryogenesis from leaf discs of *Coffea canephora*. Plant Sci. 107, 199-204.

HARI V. 1980 Effect of cell density changes and conditioned media on carrot somatic embryogenesis. Z. Pflanzenphysiol. 96, 227-231.

HELARIUTTA Y., FUKAKI H., WYSOCKA-DILLER J., NAKAJIMA K., JUNG J., SENA G., HAUSER M.T. & BENFEY P.N. 2000 The SHORT-ROOT gene controls radial patterning of the *Arabidopsis* root through radial signaling. Cell 101, 555-567.

INGRAM G., BOISNARD-LORIG C., DUMAS C. & ROGOWSKY P.M. 2000 Expression patterns of genes encoding HD-ZipIV homeo domain proteins define specific domains in maize embryos and meristems. Plant J. 22, 401-414.

IRISH V.F. & SUSSEX I.M. 1992 A fate map of the *Arabidopsis* embryonic shoot apical meristems. Development 115, 745-753.

JÜRGENS G., TORREZ RUIZ R.A. & BERLETH T. 1994 Embryonic pattern formation in flowering plants. Annu. Rev. Genet. 28, 351-357.

KERMODE A.R. 1990 Regulatory mechanisms involved in the transition from seed development to germination. Crit. Rev. Plant Sci. 9, 155-195.

KERMODE A.R., DUMBROFF E.B. & BEWLEY J.D. 1989 The role of maturation drying in the transition from seed to germination. VII Effects of partial and complete desiccation on abscisic acid levels and sensistivity in *Ricinus communis* L. seeds. J. Exp. Bot. 40, 303-313.

KOMAMINE A., MATSUMOTO M., TSUKAHARA M., FUJIWARA A., KAWAHARA R., ITO M., SMITH J., NOMURA K. & FUJIMURA T. 1990 Mechanisms of somatic embryogenesis in cell cultures - physiology, biochemistry and molecular biology. pp. 307-313 in Nijkamp H.J.J., Van der Plas L.H.W. & Van Aartrijk J. (eds.) *Progress in Plant Cellular and Molecular Biology*. Kluwer Academic Publishers, The Nether-lands.

KREUGER M. & VAN HOLST G.J. 1993 Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. Planta 189, 243-248.

KREUGER M. & VAN HOLST G.J. 1995 Arabinogalactan proteins epitopes in somatic embryogenesis of *Daucus carota* L. Planta 197, 135-141.

KREUGER M. & VAN HOLST G.J. 1996 Arabinogalactan proteins and plant differentiation. Plant Mol. Biol. 30, 1077-1086.

KREUGER M., POSTMA E., BROUWER Y. & VAN HOLST G.J. 1995 Somatic embryogenesis of *Cyclamen persicum* in liquid medium. Physiol. Plant. 94, 605-612.

KURUP S., JONES H.D., & HOLDSWORTH M.J. 2000 Interactions of the developmental regulator ABI1 with proteins identified from developing *Arabidopsis* seeds. Plant J. 21, 143-155.

KUTSCHERA U. 1994 The current status of the acid-growth hypothesis. New Phytol. 126, 549-569.

LAUX T. & JÜRGENS G. 1997 Embryogenesis. A new start in life. Plant Cell 9, 989-1000.

LI Z. & THOMAS T.L. 1998 PEI1, an embryo-specific finger protein gene required for heart-stage embryo formation in *Arabidopsis*. Plant Cell 10, 383-398.

LINDSEY K. & TOPPING J.F. 1993 Embryogenesis: a question of patterns. J. Exp. Bot. 44, 359-374.

LITZ R.E. & GRAY D.J. 1995 Somatic embryogenesis for agricultural improvement. World J. Microbiol. Biotechnol. 11, 416-425.

LOPES M.A. & LARKINS B.A. 1993 Endosperm origin, development and function. Plant Cell 5, 1383-1399.

LO SCHIAVO F., PITTO L., GIULIANO G., TORTI G., NUTI-RONCHI V., MARAZZITI D., VERAGA R., ORSELLI S. & TERZI, M. 1989 DNA methylation of embryogenic carrot cell cultures and its variations as caused by maturation, differentiation, hormones and hypomethylation drugs. Theor. Appl. Genet. 77, 325.

LO SCHIAVO F. 1994 The genetics and physiology of the totipotent cell. pp 73-84 in Couzzi G. & Puigdomenech P. (eds.) *Plant Molecular Biology*. Springer-Verlag , Berlin, Heidelberg,

LO SCHIAVO F. 1995 Early events in embryogenesis. in Bajaj Y.P.S. (ed.) *Biotechnology in Agriculture and Forestry vol 30, Somatic embryogenesis and synthetic seeds*, Springer Verlag Berlin, Heidelberg.

LOTAN T., OHTO M., YEE K.M., WEST M.A., LO R., KWONG R.W., YAMAGISHI K., FISCHER R.L., GOLDBERG R.B. & HARADA J.J. 1998 *Arabidopsis* LEAFY COTYLEDON1 is sufficent to induce embryo development in vegetative cells. Cell 93, 1195-1205.

LU P., PORAT R., NADEAU J.A. & O'NEILL S.D. 1996 Identification of a meristem L1 layer-specific gene in *Arabidopsis* that is expressed during embryogenesis and defines a new class of homeo box genes. Plant Cell 8, 2155-2168.

MAJEWSKA-SAWKA A. & NOTHNAGEL E.A. 2000 The multiple roles of arabinogalactan proteins in plant development. Plant Physiol. 122, 3-9.

MARSDEN M.P.F. & MEINKE D.W. 1985 Abnormal development of the suspensor in an embryo-lethal mutant of *Arabidopsis thaliana*. Am. J. Bot. 72, 1801-1812.

MAYER U. & JÜRGENS G. 1998 Pattern formation in plant embryogenesis: A reassessment. Semin. Cell. Dev. Biol. 9, 187-193.

MAYER U., TORREZ-RUIZ R.A., BERLETH T., MISERA S. & JÜRGENS G. 1991 Mutations affecting body organization in the *Arabidopsis* embryo. Nature 353, 402-407.

MAYER K.F., SCHOOF H., HAECKER A., LENHARD M., JÜRGENS G. & LAUX T. 1998 Role of WUSCHEL in regulating stem cell fate in *Arabidopsis* shoot meristem. Cell 95, 805-815.

McCABE P.F., VALENTINE T.A., FORSBERG L.S. & PENNELL R.I. 1997 Soluble signals from cells identified at the cell wall establish a developmental pathway in carrot. Plant Cell 9, 2225-2241.

McKERSIE B.D. & BROWN D.C.W. 1996 Somatic embryo-genesis and artificial seeds in forage legumes. Seed Sci. Res. 6, 109-126.

MEINKE D.W. 1995 Molecular genetics of plant embryogenesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 369-394.

MERKELE S.A., PARROTT W.A. & FLINN B.S. 1995 Morphogenic aspects of somatic embryogenesis. pp. 155-203 in Thorpe T.A. (ed.) *In Vitro Embryogenesis in Plants.* Kluwer Academic Publishers, Dordrecht, Boston, London.

MORDHORST A.P., TOONEN M.A. & DE VRIES S.C. 1997 Plant Embryogenesis. Crit. Revi. Plant Sci. 16, 535-576.

MORDHORST A.P., VOERMAN K.J., HARTOG M.V., MEIJER E.A., VAN WENT J., KOORNNEEF M. & DE VRIES S.C. 1998 Somatic embryogensis in *Arabidopsis thaliana* is facilitated by mutations in genes repressing meristematic cell divisions. Genetics 149, 549-563.

MURTHY B.N.S., MURCH S.J. & SAXENA P.K. 1998 Thidiazuron: A potent regulator of *in vitro* plant morphogenesis. In Vitro Cell. Dev. Biol. -Plant 34, 267-275.

NAGL W. 1976 Ultrastructural and developmental aspects of autolysis in embryo- suspensors. Ber. Dtsch. Bot. Ges. 89, 301-311.

NEALE D.B. & SEDEROFF R.R. 1989 Paternal inheritance of chloroplast DNA and maternal interitance of mitochondrial DNA in loblolly pine. Theor. Appl. Genet. 77, 212-216.

NOMURA K. & KOMAMINE A. 1985 Identification and isolation of single cells that produce somatic embryos at a high frequency in a carrot suspension culture. Plant Physiol. 79, 988-991.

OSUGA K., MASUDA H. & KOMAMINE A. 1999 Synchroniz-ation of somatic embryogenesis at high frequency using carrot suspension cultures: model systems and application in plant development. Plant Mol. Biol. 21, 129-140.

OTEGUI M. & STAEHELIN L.A. 2000 Syncytal-type cell plates: A novel kind of cell plate involved in endosperm cellularization of *Arabidopsis*. Plant Cell 12, 933-947.

OWENS J.N. & MOLDER M. 1984 *The reproductive cycle of Interior spruce.* Information Service Branch, B.C., Ministry of Forest, Victoria, B.C, Canada.

PARCY F., KOHARA A., MISERA S. & GIRAUDAT J. 1997 The ABSCISIC ACID-INSENSITIVE3, FUSCA3 and LEAFY

COTYLEDON1 loci act in concert to control multiple aspects of *Arabidopsis* seed development. Plant Cell 9, 1265-1277.
POLLOCK B.M. 1969 Imbibition temperature sensitivity of Lima beans controlled by initial seed moisture. Plant Physiol. 44, 907-911.
RAGHAVAN V. & SHARMA K.K. 1995 Zygotic embryogenesis in gymnosperms and angiosperms. pp 73-115 in Thorpe T.A. (ed.) *In vitro Embryogenesis in Plants*. Kluwer Academic Publishers, Dordrecht, Boston, London.
RAINS D.W. 1989 Plant tissue and protoplast culture: Applications to stress physiology and biochemistry. pp. 181-196 in Jones H.G., Flowers T.J. & Jones M.B. (eds.) *Plants and Stress*. Cambridge University Press. ISBN 0-521-34423-9.
REINERT J. 1958 Untersuchungen über die Morphogenese an Gewebenkulturen. Ber. Dtsch. Bot. Ges. 71, 15.
ROBERTS D.R., SUTTON B.C.S. & FLINN B.S. 1990 Synchronous and high frequency germination of interior spruce somatic embryos following partial drying at high relative humidity. Can. J. Bot. 68, 1086-1090.
ROMBERGER J.A., HEJNOWICZ Z. & HILL J.F. 1993 The embryo. In: *Plant structure: function and development* Springer-Verlag, Berlin, Heidelberg.
RUSSELL S.D. 1993 The egg cell: development and role of in fertilization and early embryogenesis. Plant Cell 5, 1349-1359.
SABALA I., ELFSTRAND M., FARBOS I., CLAPHAM D. & VON ARNOLD S 2000 Tissue-specific expression of *Pa18*, a putative lipid transfer protein gene, during embryo development in Norway spruce (*Picea abies*). Plant Mol. Biol. 42, 461-478.
SAMAJ J., BALUSKA F., BOBAK M. & VOLKMANN D. 1999 Extracellular matrix surface network of embryogenic units of friable maize callus contains arabinogalactan-proteins recognized by monoclonal antibody JIM4. Plant Cell Rep. 18, 369-374.
SATOH N., HONG S.-K., NISHIMURA A., MATSUOKA M., KITANO H. & NAGATO Y. 1999 Initiation of shoot apical meristem in rice: characterization of four SHOOTLESS genes. Development 126, 3629-3636.
SCHOOF H., LENHARD M., HAECKER A., MAYER K.F.X., JÜRGENS G. & LAUX T. 2000 The stem cell population of *Arabidopsis* shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. Cell 100, 635-644.
SENTOKU N., SATO Y., KURATA N., ITO Y., KITANO H. & MATSUOKA M. 1999 Regional expression of the rice KN1-type homeobox gene family during embryo, shoot, and flower development. Plant Cell 11, 1651-1663.
SHELLHAMMER J. & MEINKE D.W. 1990 Arrested embryos from the bio1 auxotroph *Arabidopsis thaliana* contain reduced levels of biotin. Plant Physiol. 93, 1162-1167.
SHEVELL D.E., LEU W.-M., GILLMOR C.S., XIA G., FELDMANN K.A. & CHUA N-H. 1994 *EMB30* is essential for normal cell division, cell expansion, and cell adhesion in *Arabidopsis* and encodes a protein that has similarity to Sec7. Cell 77, 1051-1062.
SINGH H. 1978 Embryology of gymnosperms. pp. 187-241 in Zimmerman W., Carlquist Z., Ozenda P. & Wulff H.D. (eds.) *Handbuch der Pflanzenanatomie*. Gebrüder Borntraege, Berlin, Stuttgart.
SMITH D.L. & KIRKORIAN A.D. 1990 Somatic proembryo production from excised, wounded zygotic carrot embryos on hormone-free medium: evaluation of the effects of pH, ethylene and activated charcoal. Plant Cell Rep. 9, 468-470.
SMITH L.G., JACKSON D., & HAKE S. 1995 Expression of KNOTTED1 marks shoot meristem formation during maize embryogenesis. Dev. Gen. 16, 344-348.
SMITH J.A. & SUNG Z.R. 1985 Increase in regeneration of plant cells by cross feeding with regenerating *Daucus carota* cells. pp. 133-136 in Terzi M., Pitto L. & Sung Z.R. (eds.) *Somatic Embryogenesis*. Incremento Produttivita Risorse Agricole, Rome.
SOMLEVA M.N., SCHMIDT E.D.L. & DE VRIES S.C. 2000 Embryogenic cells in *Dactylis glomerata* L. (Poaceae) explants identified by cell tracking and by SERK expression. Plant Cell Rep. 19, 718-726.
SPAINK H.P., SHEELEY D.M., VAN BRUSSEL A.A.N., GLUSHKA J., YORK W.S., TAK T., GEIGER O., KENNEDY E.P., REINHOLD V.N. & LUGTENBERG B.J.J. 1991 A novel highly unsaturated fatty acid moiety of lipooligosacharide signals determines host specificity of *Rhizobium*. Nature 354, 125-130.
STEINMANN T., GELDNER N., GREBE M., MANGOLD S., JACKSON C.L., PARIS S., GALWEILER L., PALME K. & JÜRGENS G. 1999 Coordinated polar localisation of auxin efflux carrier PIN1 by GNOM ARF GEF. Science 286, 316-318.
STEWARD F.C., MAPES M.O. & HEARS K. 1958 Growth and organized development of cultured cells. II. Growth and division of freely suspended cells. Am. J. Bot. 45, 705-708.
SVETEK J., YADAV M.P. & NOTHNAGEL E.A. 1999 Presence of glycosylphosphatidylinositol lipid anchor on rose arabinogalactan proteins. J. Biol. Chem. 274, 14724-14733.
THOMAS T.L. 1993 Gene expression during plant embryogenesis and germination: An overview. Plant Cell 5, 1401-1410.
THOMAS W., HOFFMANN F., POTRYKUS I. & WENZEL G. 1976 Protoplast regeneration and stem embryogenesis of haploid androgenic rape. Mol. Gen. Genet. 145, 245-247.
THOMPSON H.J.M. & KNOX J.P. 1998 Stage-specific responses of embryogenic carrot cell suspension cultures to arabinogalactan protein-binding β–glucosyl Yariv reagent. Planta 205, 32-38.
TOONEN M.A.J., HENDRIKS T., SCHMIDT E.D.L., VERHOEVEN H.A.,VAN KAMMEN A. & DE VRIES S.C. 1994 Description of somatic-embryo-forming single cells in carrot suspension cultures employing video cell tracking. Planta 194, 565-572.
TOONEN M.A.J. & DE VRIES S.C. 1997 Use of videl tracking to identify embryogenetic cultured cells. pp 1-45 in Lindsey K. (ed.) *Plant Tissue Culture Manual*. Kluwer Academic Publishers, Dordrecht. The Netherlands.
TRUCHET G., ROCHE P., LEROUGE P., VASSE J., CAMUT S., DE BILLY F., PROME J.-C. & DENARIE J. 1991 Sulfated lipo-oligosacharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature 351, 670-673.
TSUKAHARA M. & KOMAMINE A. 1997 Separation and analyses of cell types involved in early stages of carrot somatic embryogenesis. Plant Cell Tissue Organ Cult. 47, 145-151.
VAN HENGEL A.J. 1998 *Chitinases and arabinogalactan proteins in somatic embryogenesis*. PhD thesis. Wageningen Agricultural University, Wageningen, The Netherlands.
VAN HENGEL A.J., GUZZO F., VAN KAMMEN A. & DE VRIES S.C. 1998 Expression pattern of the carrot EP3 endochitinase genes in suspension cultures and in developing seeds. Plant Physiol. 117, 43-53.
VERTUCCI C.W. 1989 The kinetics of seed imbibition: Controlling factors and relevance to seedling vigor. pp 93-115 in Stanwood P.C. & MacDonald M.B. (eds.) *Seed Moisture*. CSSA Special Publication no 14. Crop Science Society of America, Madison, WI. ISBN 0-89118-525-9.
VERNON D.M. & MEINKE D.W. 1994 Embrygenic transformation of the suspensor in twin, a polyembryonic mutant of *Arabidopsis*. Dev. Biol. 165, 566-573.
VIELLE-CALZADA, J.-P., BASKAR R. & GROSSNIKLAUS. 2000 Delayed activation of the paternal genome during seed developmant. Nature 404, 91-94.
WEHMEYER N. & VIERLING E. 2000 The expression of small heat shock proteins in seeds responds to discrete developmental

signals and suggests a general protective role in desiccation tolerance. Plant Physiol. 122, 1099-1108.
WEST M.A.L. & HARADA J.J. 1993 Embryogenesis in higher plants: an overview. Plant Cell 5, 1361-1369.
WILLIAMS E.G. & MAHESWARAN G. 1986 Somatic embryogenesis: Factors influencing coordinated behavior of cells as an embryogenic group. Ann. Bot. 57, 443-462.
YADEGARI R., DE PAIVA G.R., LAUX T., KOLTUNOW A.M., APUYA N., ZIMMERMAN J., FISHER R.L., HAADA J.J. & GOLDBERG R.B. 1994 Cell differentiation and morphogenesis are uncoupled in *Arabidopsis* raspberry embryos. Plant Cell 6, 1713-1729.
YAKOVLEV M.S. & YOFFE M.D. 1957 On some peculiar features in the embryogeny of *Paeonia* L. Phytomorphology 7, 74-82.
YEUNG E.C. 1995 Structural and developmental patterns in somatic embryogenesis. pp. 205-247 in Thorpe T.A. (ed.) *In vitro Embryogenesis in Plants*. Kluwer Academic Publishers Dordrecht, Boston, London.
YEUNG E.C & MEINKE D.W. 1993 Embryogenesis in angiosperms: development of the suspensor. Plant Cell 5, 1371-1381.
ZHANG J. Z. & SOMERVILLE C. R. 1997 Suspensor-derived polyembryony caused by altered expression of valyl-tRNA synthetase in the *twin2* mutant of *Arabidopsis*. Proc. Natl. Acad. Sci. USA 94, 7349-7355.
ZIMMERMAN J.L. 1993 Somatic embryogenesis: A model for early development in higher plants. Plant Cell 5, 1411-1423.

Chapter 10
Adventitious Regeneration

1. INTRODUCTION

New organs such as shoots, roots or embryos can be induced to form on plant tissues lacking pre-existing meristems. Such freshly originated organs are said to be adventive or adventitious. The creation of new form and organisation, where previously it was absent, is termed adventitious organogenesis, or, in the case of embryo-formation, somatic embryogenesis.

Adventitious formation of shoots is referred to as **caulogenesis** and of roots as **rhizogenesis**.

From explants, structures may be formed that are similar to the embryos found in true seeds. Such embryos often develop a region equivalent to the suspensor of zygotic embryos and, unlike shoot or root buds, come to have both a shoot and a root pole. To distinguish them from zygotic or seed embryos, embryos produced from cells or tissues of the plant body are called somatic embryos and the process leading to their inception is termed **somatic embryogenesis** (see Chapter 9). The word 'embryoid' has been especially used when it has been unclear whether the embryo-like structures seen in cultures were truly the somatic equivalent of zygotic embryos. Somatic embryogenesis is now such a widely observed and documented event that somatic embryo has become the preferred term. The formation of leaves adventitiously *in vitro* usually denotes the presence of a shoot meristem. Sometimes leaves appear without apparent shoot formation: opinions are divided on whether such leaves can have arisen themselves *de novo*, or whether a shoot meristem must have been present first of all and subsequently failed to develop. An example is regeneration from flower-stem explants in tulip (Kuipers and Langens-Gerrits, 1997). The formation of flowers or floral parts is rare, occurring only under special circumstances. They are formed from, e.g., flower stem tissues (Van Den Ende *et al.,* 1984a,b). This type of regeneration is not relevant to plant propagation.

Caulogenesis, rhizogenesis and somatic embryogenesis are essential for plant multiplication *in vitro*. New plants are seldom obtained in culture by the union of shoots and roots independently formed in callus. The production of complete plantlets by adventitious regeneration is achieved from adventitious shoots, which are subsequently rooted, or from somatic embryos. Shoots, roots and somatic embryos arise from single cells, or groups of cells, which become induced by the cultural conditions to become centres of active cell division (adventitious meristems), each capable of producing an organ of the one kind.

Regeneration has now been observed *in vitro* in numerous plants of many genera, but it cannot yet be induced universally. Even within a single species, varieties can be found, which are recalcitrant. Moreover, the capacity for regeneration can be lost after transformation e.g. the callus of *Pisum sativum* that becomes recalcitrant on transformation (Nauerby *et al.,* 1990;1991)

Adventitious meristems can theoretically occur in either of two distinct ways:

- Directly from the differentiated cells in a newly-transferred piece of whole-plant tissue, without intermediate proliferation of undifferentiated tissue;
- Indirectly from the unspecialised, unorganised and dedifferentiated cells of callus tissues or suspension cultures.

Hicks (1980) described these two methods of regeneration as direct and indirect organogenesis respectively. In practice, it is not always possible to distinguish between the two methods. Directly-formed meristems destined to become shoots or somatic embryos may proliferate to form a regenerative tissue similar in appearance to callus. Such meristems may also become surrounded by dedifferentiated callus so that it is difficult to ascertain their origin. A type of callus bearing superficial shoot-forming meristems can often be separated from this kind of culture. Alternatively, shoot meristems may form within callus which is still attached to, or within the tissues of the primary explant, or may be produced simultaneously from such callus and from superficial cells of the explant (Bigot *et al.,* 1977).

1.1. COMPETENCE AND DETERMINATION

Highly specialised cells of the intact plant are never observed to change from their existing differentiated state. In addition, there are some cells, which retain the capacity to divide yet never form organs and are considered to be recalcitrant. Similarly, from some cells new organs are never formed so they are thought to have lost the capacity

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to form new plants. Cells, which have retained the capacity for a particular kind of cellular differentiation or regeneration, or have acquired it in response to an appropriate stimulus, are said to be competent.

Competence may be defined as the state of a cell in which it is able to respond to extra-cellular signals. Determination then becomes the state of a previously competent cell that has responded to the external signal so committing it to a differentiation pathway which will include organogenesis. If cells of an explant are not already competent at the time of excision, they may be induced to become so *in vitro*. They are then enabled to respond to the organogenetic stimulus provided by a particular combination of growth regulators (possibly different to those required for the induction of competence) added to the medium.

1.2. REGENERATION AND CELLULAR DIFFERENTIATION

Thus, development of competence is the first step in the dedication of one or more cells (Fig. 10.1, Stage 1) towards regeneration, or some other kind of specialised development (Fig. 10.1, Stage 2). In this scheme, a cell in the ground state will have maintained its original capacity either of competence or recalcitrance. If the latter, competence will need to be induced. The second stage of dedication is then said to be the induction of determination in competent cells. Individual cells or groups of cells are said to be determined when they have become irreversibly committed to follow a particular genetically-programmed developmental pathway, for example a particular kind of regeneration (Fig. 10.1, Stage 3) and can continue towards that outcome without the further influence of growth regulators required during the previous induction stage (Christianson, 1987). Determined cells differentiate to become the specialised component tissues of mature plants.

Although growth regulators may also help to induce direct regeneration, cells in some parts of a plant appear to be partially pre-determined to a particular morphogenetic pathway so that it takes only a slight change in environment to induce the tissues of some explants to form an adventitious meristem instead of progressing to become a differentiated cell within the intact plant. This is clearly seen in tissues that are already embryogenically committed. There is a high probability that somatic embryogenesis can be initiated from such tissues when they are explanted, either directly, or indirectly after some initial cell proliferation.

Regeneration from cells, which are already committed to such development has been called permissive, while that from cells induced to become morphogenetic by endogenous or exogenous growth regulators, inductive (Smith and Krikorian, 1988). Thus, permissive and inductive regeneration have been considered to be almost synonymous with direct and indirect regeneration

Morphogenetic centres arise from only a relatively small proportion of the total number of cells in a culture. It is not known why this should be. In many circumstances it would clearly be advantageous to be able to obtain increased numbers. The extent of pre-determination or commitment to regeneration varies between different tissues of the whole plant; that of any given tissue will differ according to the plant's genetic background and the position of the tissues within that plant.

Some observers believe that the cells from which organ-producing meristems are formed, are the only ones in a cell population that are competent. An alternative hypothesis was put forward by Street (1977), who suggested that although many cells may have the capacity to become competent, newly formed meristems may preferentially accept essential metabolites from surrounding cells which would then be less likely to follow the same developmental pathway. There is also evidence (for example, Cassells, 1979) that additional meristems are primarily inhibited from developing by growth substances produced by the first meristems to be initiated in the tissue. This would indicate that cells that preferentially give rise to meristems, do so because they become induced to differentiate slightly before their competitors. They may, for instance, be at an appropriate phase of the cell cycle at the time an inductive stimulus is applied. It is likely that cells will have exited from the cell cycle on differentiating (specialising) and can be considered to be in G0. In this state, they will not be able to receive signals inducing them to differentiate in a particular direction. They must re-enter G1 during which period of the cell cycle there is a window of time in which they may receive a signal to become competent/differentiate (Roberts *et al.,* 1988). It is possible that the process of re-entry to the cell cycle constitutes the process of acquiring competence in that such cells can then respond to the signals for differentiation. Not all cells will be able to re-enter

the cell cycle at G1. Thus, within the concept of differentiated cells in the explant are those which are fully differentiated (highly specialised) e.g. xylem and phloem, and those which are differentiated in the sense that they are specialised for a particular function yet retain the capacity for cell division e.g. cortical parenchyma, collenchyma. The latter cells will be able to re-enter the cell cycle and so become competent and be determined for an alternate form of differentiation, the former will not.

1.3 THE EPIGENETIC NATURE OF COMMITMENT

A commitment to progress towards a defined developmental objective, means that a cell will follow a defined programme of development. Commitment is self-maintained in that it can be passed on to daughter cells and later cell generations. The message is not immutable though, and may be lost or changed by subsequent stimuli (Wolffe and Matzke, 1999). Determination usually results in differentiated cells (Fig 10.1, Stage 4), which retain the developmental determination of their parents, and with their differentiated state being replicated during cell division. When morphogenetic determination or cell differentiation becomes fixed, or irreversible, it is said to have become canalized.

Several kinds of morphogenetic determination are possible. Cells may become committed to produce root, shoot, embryo, leaf or floral meristems. Once canalization has occurred, progress through a single development pathway is then usually inevitable unless the cells are capable of being stimulated to divide under conditions which promote dedifferentiated growth.

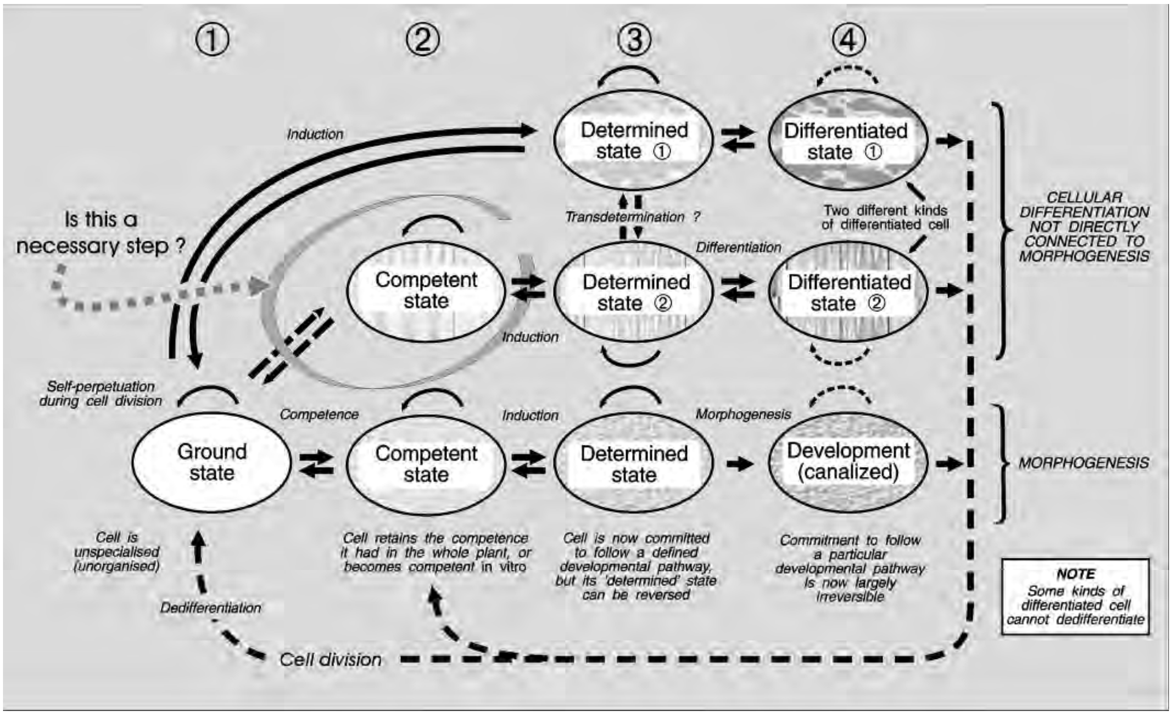

Fig. 10.1 Progressive steps in the capacity of a cell to become differentiated and/or morphogenic. Competence can be one step on the pathway to determination, but it is not clear whether cells in the ground state always have to pass through this stage as they are committed to development.

However, at an early stage of development, leaf and floral meristems can sometimes be induced to differentiate into shoots without dedifferentiation occurring. Some kinds of canalized development (e.g. the formation of tracheids) are irreversible.

The mechanisms by which the growth regulators influence cells in explants may be considered at five levels. They are:

1. the amounts of endogenous growth regulators present in the explants,
2. the amounts of the exogenously applied growth regulators entering the explant,

3. the pathways by which the growth regulators pass through the cells of the explants,
4. the point at which they may signal events in the cells concerned and
5. the pathways by which these signals may reach the genes and so have their effect.

Some measurements have been made to determine the amounts of endogenous growth regulators present in explants and can vary from femtomol to picomol levels per unit of measurement (e.g. per cell, per µg protein), depending upon the type of explant measured (Gahan *et al.,* 1995,1996; Wyndaele *et al.,* 1988; Reuther *et al.,* 1996). However, such measurements from whole explants do not indicate the distribution of the growth regulators between cells and even the levels in each cell at particular stages of the cell cycle although studies by Redig *et al.* (1996) have shown the mean values for levels of each type of cytokinin to vary at each stage of the cell cycle in single cell cultures of synchronized tobacco BY-2 cells.

Although the literature contains many methods for the induction of organogenesis for many explants, the optimal amounts of growth regulators added to the culture medium are comparatively large, yet there are very few measurements as to how much of the applied auxin or cytokinin actually enters the explant with time of culture. Those that have been made indicate that a low amount of the growth regulator enters the explant and may even reach an equilibrium at these low levels (e.g. Gahan *et al.,* 1995,1998; Wang *et al.,* 2001; Reuther *et al.,* 1998; Ross and Zoglauer, 1998).

Having entered the explant, the growth regulators move through the cells and tissues, presumably creating gradients. Although it would be expected that they would pass through the tissues via the apoplastic pathway (in between the cells), it is possible that both the apoplastic and symplastic pathways (through the cells) are initially involved. This has a bearing on the signalling mechanisms that might be involved. Thus, if the growth regulators move by the apoplastic pathway, then signal transduction might be expected to pass via the plasmalemma. If the symplastic pathway is involved, it is possible that signalling may occur directly in the nucleus. Evidence from tomato cotyledon explants indicates that the initial movement of cytokinin and auxin from the medium into the explant may be via both pathways (Wang *et al.,* 2001) when apoptotic changes are induced in the epidermis and subepidermal cells adjacent to the medium. In this case, the signalling by cytokinin may be directly in the nucleus, nuclear cytokinin-binding has been shown to occur in *Nicotiana tabacum* (Dewitte *et al.,* 1999) with the cytokinin acting as would an oestrogen in an animal cell. Having passed through the initial cell layers of the explant, an apoplastic pathway could then take over which would involve signalling pathways *via* the plasmalemma (see Francis *et al.,* 1998; Sitaramayya, 1999).

Plasmamembrane signalling by auxin is problematic. The auxin-binding protein, ABP1, was shown to be present in a number of species, the ABP1s from both maize and tobacco being immunologically related, present on the exterior face of the plasmamembrane, and coupled to an H^+ ATPase (Macdonald 1997; Venis and Napier, 1997). They appeared to be essential for the auxin response and have been linked to G-protein signalling, possibly via IP3, (Macdonald, 1997; Hobbie, 2003). Protein kinases have also been implicated in auxin signal transduction (Hobbie, 2003). Although there is a functional pool of ABP1 on the outer surface of the plasmamembrane, there appear to be too few receptor molecules for there to be adequate signalling sites for auxin action. More recently (Dharmasiri, *et al.*, 2005; Kepinski *et al.*, 2005; Woodward *et al,* 2005) have shown that auxin binds directly to a protein, TIR1, which in turn is triggered to bind to a class of proteins Aux/IAA. The latter are known gene repressors. The auxin-TIR1 complex initiates the attachment of ubiquitin to Aux/IAA proteins so identifying them for destruction when the genes they repress are activated.

Cytokinin signalling systems are more clearly defined. The data of Hooley (1998) and Plakidou-Dymock *et al.* (1998) have shown that the BA could be signalling via GCR1, a plant homologue of the animal cell GPCR, a seven-pass plasmamembrane-linked protein, through the G-protein signalling pathways. The components of the G-protein linked secondary messenger systems known for animal cells have been demonstrated to be present, in the main, in higher plant cells. Furthermore, the identification of an histidine kinase gene *CKI1* which induces typical cytokine responses (Kakimoto *et al.,* 1996) has led to the isolation of a cytokinin receptor gene *CRE1* from *Arabidopsis thaliana* (Inoue *et al.,* 2001). *CRE1* codes for an histidine kinase which appears to initiate phospho-relay signalling (see in Francis *et al.,* 1998; Sitaramayya, 1999; Nogué *et al.,* 2003) (For more information on signalling see Chapters 5 to 7).

2. WOUNDING

In the natural environment the wounding of plants by pruning, grazing or mechanical abrasion often stimulates callus formation and/or regeneration. All explants transferred to culture are subjected to wounding during their isolation. The smaller the explant, the greater the proportion of wounded surface. In many instances, damage causes the release of inhibitory substances (see Chapter 11), but it also induces the natural production of the gaseous hormone ethylene, which frequently provides a stimulus to the formation of unorganised tissue or adventitious shoots and roots. The physiological changes in tissues, which bring about these responses (the so-called wound reaction) are discussed in other sections of the book. The advantage to be gained from wounding can be such that explants are sometimes given additional damage, above that induced by excision (e.g. leaves of *Coronaria varia* scratched or peeled before incubation to induce callus formation - Mariotti and Arcioni, 1983).

2.1. WOUNDING DURING ISOLATION

Regeneration frequently occurs at the cut surface of explants. On cotyledons from *Pyrus malus* seed embryos which were cut into halves, the production of a small amount of callus, followed by multiple adventitious shoots, occurred more frequently from the distal cut surface of proximal sections, than on the proximal end of distal sections (Browning *et al.,* 1987; Fig. 10.2). Although the cut edges of *Ipomoea batatas* leaves did not form embryogenic callus, it was formed on the cut edges of stem and leaf explants (Liu and Cantcliffe, 1984). Fragmentation of young expanding leaves of *Malus* into 14 sections improved the capacity of the leaf tissue to form adventitious shoots and embryo-like structures (Welander, 1988).

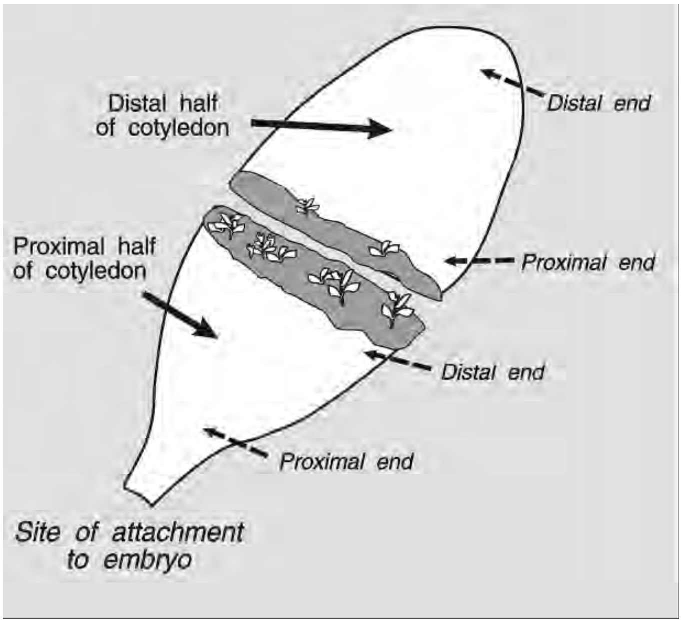

Fig. 10.2 The polarity of adventitious shoot formation on *Pyrus malus* cotyledon pieces [after Browning *et al.,* 1987].

If cultured intact, all embryo axes of the apple cultivar ‘Delicious’ and 55% of those of cv. ‘Winesap’ formed only callus, but if cut into 3 roughly equal sections (root, hypocotyl and plumule), a high proportion of the plumule and hypocotyl pieces of both cultivars formed adventitious shoots. None of the embryo root sections of ‘Delicious’ formed shoots and only 11% of ‘Winesap’ ones. Korban and Skirvin (1985) suggested that sectioning had either removed a factor, which was inhibiting regeneration (possibly one contained in the root

zone), or had increased the activity of a promotory substance.

Some explants are insensitive to treatment with growth regulators unless they are damaged or sectioned. Axillary buds (areoles) of the cactus *Opuntia polycantha* were insensitive to auxin or cytokinin in the medium unless they were wounded in some way. Then the majority formed adventitious roots in response to 10 mg/l NAA and shoots in response to 10 mg/l BA (Mauseth and Halperin, 1975). In some species, splitting a shoot tip explant in half along its longitudinal axis can practically double the number of axillary shoots produced (e.g. in *Yucca elephantipes*, Bentz *et al.,* 1988).

2.2. ADDITIONAL WOUNDING

Inflicting further wounds to an explant after excision can often increase regeneration. For example, cuts across the leaf lamina and main veins of mature phase leaf explants of *Liquidambar styraciflua* var. 'Mariane' significantly increased the number of adventitious shoots produced per explant. The treatment was not effective in the variety 'Variegata' which was naturally highly organogenetic (Brand and Lineberger, 1986).

Regeneration usually occurs at the site of the wounds. Thus the increased adventitious shoot formation on wounded leaves taken from proliferating shoot cultures of *Vaccinium corymbosum* mainly occurred along points of injury (Callow *et al.,* 1988). Park and Son (1988) noted that leaves removed from cultured shoots of *Populus nigra* and *P. maximowiczii*, formed adventitious shoots or roots on their outer surfaces, depending on the growth regulators in the medium. However, if the leaves were punctured in several locations (five was optimal) with a pin, regeneration occurred at the margins of the perforations. The authors suggested that wounding promoted the transfer of endogenous hormones to the location of the scarring, causing there to be a more suitable level of growth promotors for regeneration. This hypothesis seems to agree with results of (Norizaku *et al.,* 1985): although injury did not increase the uptake of BA into leaf sections of *Torenia fournieri*, transverse cuts across explants cultured in the presence of this regulant, significantly increased the formation of buds within 0.5 cm of the site. Longitudinal (transverse) cuts caused buds to be produced over the entire surface of the explant: without wounding they occurred mainly at its cut ends. However, wounding clearly permits an increased amount of exogenously applied growth regulators to enter *via* the wound. This can be seen when cotyledons of *Solanum aviculare* and tomato are kept in the dark so decreasing endogenous levels of both IAA and cytokinins. This results in the cells adjacent to the wounds forming vessels when in contact with 2,4D and BA (Gahan *et al.,* 1995, Wang *et al.,* 2002).

In some *Lachenalia* hybrids, bud formation on the adaxial surface of leaf sections was promoted by two superficial transverse cuts in the centre of either the adaxial or abaxial sides. Buds invariably occurred on the distal side of the cut (Niederwieser and Vcela, 1990).

2.3. ROOT FORMATION

The improved formation of adventitious roots on cuttings, which can result from wounding is described in Chapter 11.

3. THE INDUCTION OF DETERMINATION

The commitment of cells is usually modified through alterations in the *in vitro* environment, particularly by changing the availability of plant growth regulators. For regeneration to be possible, cells must be able to respond to growth regulating chemicals, that is to say they must be competent. The nature of the internal information factors governing cell competence are largely unknown. These may include natural growth substance levels within cells at the time of excision and the capacity of cells to synthesize growth substances or essential metabolites, though they may still being influenced by exogenously applied growth regulators.

3.1 COMPETENCE

In tissues of some plants, competence can be demonstrated by having two media, one without growth regulators (basal medium, BM) and the other containing growth regulators known to cause shoot formation (shoot inducing medium, SIM). In the experiments of Attfield and Evans (1991), after a varying period of culture on BM, *Nicotiana tabacum* leaf segments were transferred for induction on SIM for a specified time, and were then cultured (or re-cultured) on BM for 15 d until shoots could be counted. Explants never produced shoots if left on BM, but the longer they were on this medium (MS medium), the shorter was the period they required on

SIM (MS medium plus 5 µM BA) for shoot induction to occur.

The most rapid way to induce any given degree of shoot formation was to place the explants directly onto SIM, or give them a short 1-2 day exposure to basal medium first of all (Fig. 10.3). This indicates that 2 days was the minimum time the explants required to gain competence and that growth regulants were not required during this period. Keeping them on basal medium longer than this to begin with, lessened the exposure to BA, which was necessary to induce determination, but increased the total medium plus growth regulator induction time for determination. However, competence can be gained on media with or without growth regulants: e.g. BM, SIM, a root-inducing medium, or a callus-inducing medium (Christianson and Warnick, 1985). Thus, the induction of regeneration seems to depend on the combined effect of the medium, with or without added growth regulators, (competence) and the presence of the correct combination of growth regulators (determination). Hence, if competence requires the cell to be in the cell cycle for determination to occur, then the initial culture period (either in the presence or absence of applied growth regulators) must involve processes which result in cells moving from G0 to G1 (Roberts *et al.,* 1988).

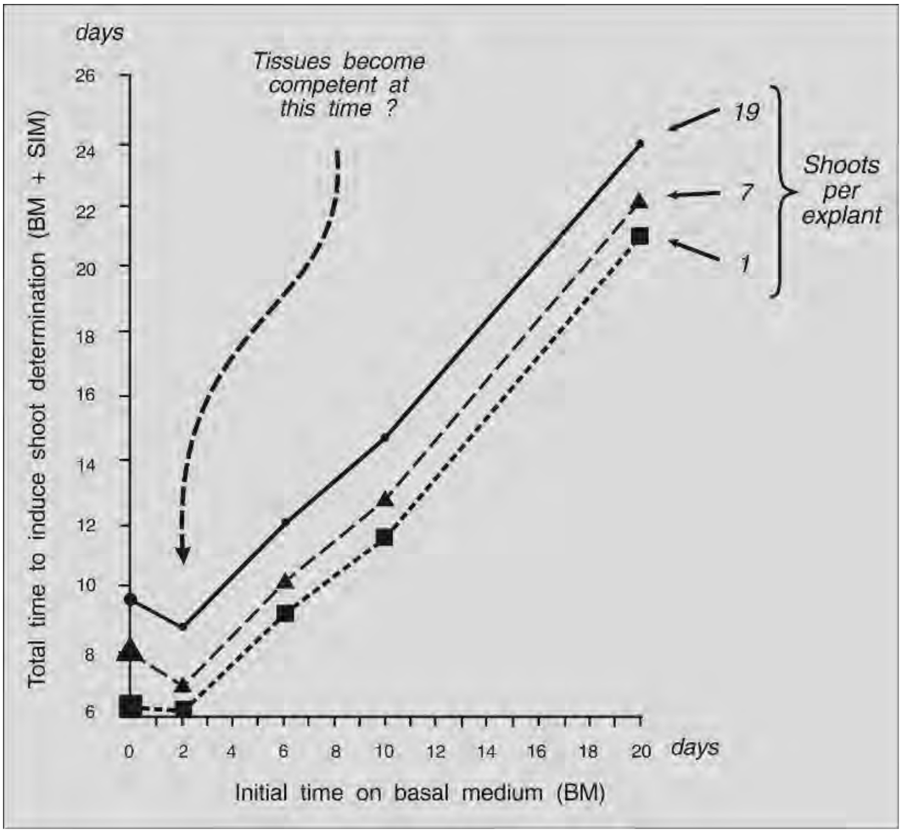

Fig. 10.3 The development of competence in *Nicotiana tabacum* leaf segments [after Attfield and Evans, 1991]. The total time to induce shoot formation was shortened by preculture on BM for more than 2 days, indicating that the tissues became competent during the initial 2-day period.

Tissues of different plants vary in their competence. Direct or indirect regeneration occurs readily only in some plant species, or may even be restricted to certain varieties within species. Changing the medium, and /or the growth regulators added to it, may induce regeneration in recalcitrant tissues, but there are still some kinds of plants in which it has not been satisfactorily achieved. An alternative explanation may lie in the silencing of relevant genes in the heterochromatin. Thus, in competent cells and tissues, the standard growth regulator treatment will signal cells to re-enter the

cell cycle from G0 (if necessary) and to proceed to G1 where they can receive the signals to differentiate. In some cases there may be a need for a pretreatment to aid the movement from G0 to G1 (e.g. an auxin shock), the relevant genes being in the euchromatic region of the chromatin. However, in recalcitrant tissues, the genes required for the passage from G0 to G1 and from G1 to S in the cell cycle will be locked in the heterochromatin and so silenced (Gahan, 2006). In this way, in the former situation, there is no recalcitrance and the cells respond directly to the growth regulators, with or without a pretreatment,. In the latter situation, the genes are silenced and unable to respond until they can be removed from the heterochromatic arrangement to the euchromatin.

The part of a plant from which an explant is derived has a major influence on direct organ formation or on the capacity of derived callus tissues to undergo regeneration. This indicates possible mechanisms for genetic control, i.e. that competence: results from the cells moving from G0 to G1 of the cell cycle when they are able to receive signals affecting genes involved in determination.

Several callus strains differing both morphologically and with regard to morphogenetic capacity may sometimes arise from one explanted piece of tissue (Fig. 10.4). Street (1979) suggested that this might happen either because cell layers or tissues varied in their morphogenetic competence as they occurred on the plant, or because competence was induced during callus formation in only certain cells. In these circumstances, callus cultures may consist of morphogenetically competent and non-competent cells or zones. Failure to isolate a competent cell line may result in its disappearance by the overgrowth of non-competent tissues.

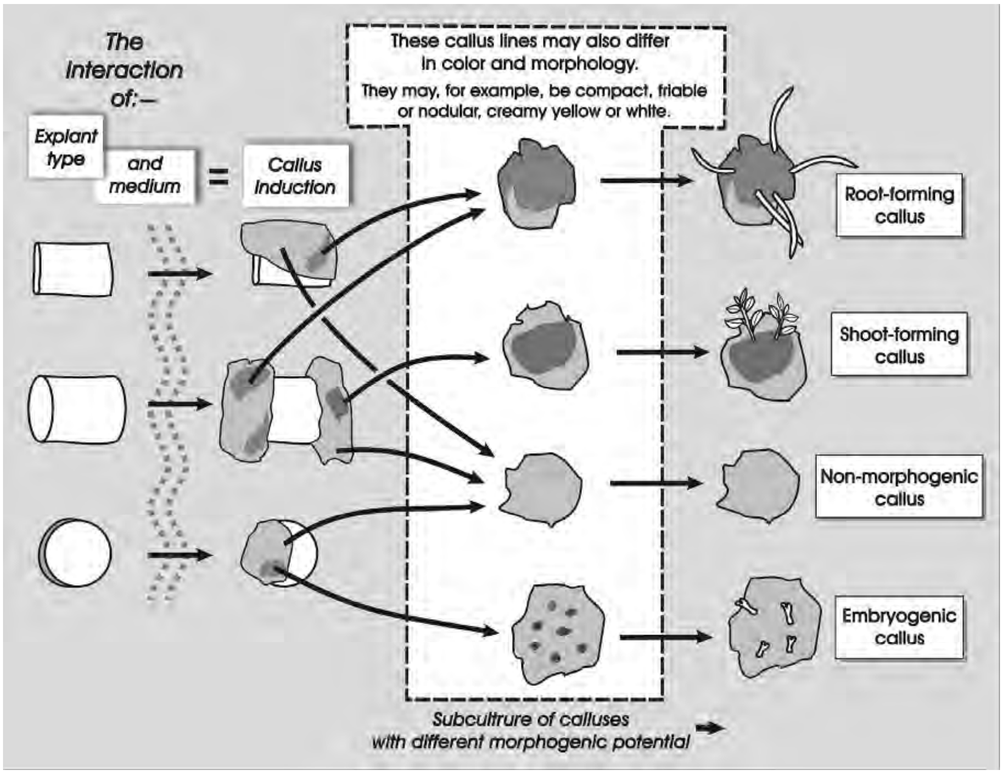

Fig. 10.4 Callus with different morphogenic potential is often isolated from a single explant.

It has also been shown that adjacent tissue layers can have correlative effects on each other. When intact stem segments of *Torenia fournieri* were cultured by Chlyah (1974), the sub-epidermal parenchyma showed no organogenetic capacity; root initials arose from cells surrounding the vascular bundles. However, if cultured alone in an appropriate medium, the sub-epidermal layers were able to initiate roots, and if they were cultured when only in contact with the stem epidermis (the other tissues having been removed), they formed shoot primordia.

3.2. DETERMINATION

Morphological determination is found not to be an 'all or nothing' event: the longer tobacco explants of Attfield and Evans (1991) were kept on SIM (up to 12 days) before transfer to BM, the more shoots they produced. Either more cells became determined as the treatment was continued or determined cells were more able to proliferate (Fig. 10.5). Shoot formation in *Pinus strobus* cotyledons was also increased the longer they were exposed to BA (5-50 µM) (up to 21 days), but with these explants a marked reduction in shoot formation occurred when they were pre-cultured for even 3 days on a basal medium and after 14 days of pre- culture they had lost their competence to produce shoots (Biondi and Thorpe, 1982; Flinn *et al.,* 1988). In this case the cells of the explant were already competent when transferred to the medium. The commitment of the *Nicotiana* explants of Attfield and Evans (1991) to produce roots was also reduced the longer they were cultured on basal medium before being transferred to a root-inducing medium (RIM). A progressively smaller number of roots was initiated, presumably because a smaller number of sites was influenced, due to a progressive decrease in the number of competent cells. Thus, although determination took place on RIM after 10 days pre-culture in basal medium, only ca. 4 roots were produced per explant, compared with 35-40 roots when the explants were placed into RIM immediately they were excised.

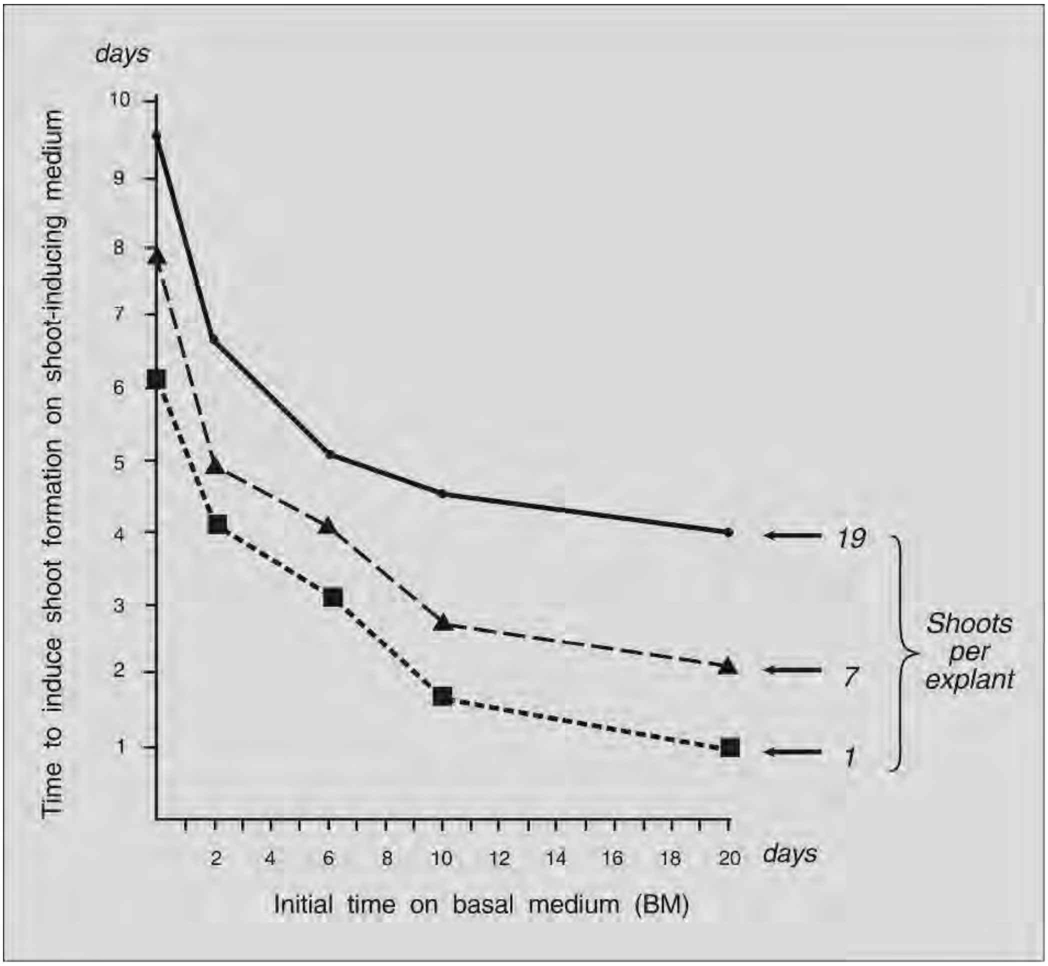

Fig. 10.5 The effect of period of exposure of *Nicotiana tabacum* leaf segments to SIM on the number of shoots produced [after Attfield and Evans, 1991]. The longer shoot segments were retained on BM, the shorter was the subsequent exposure to SIM necessary to induce any given degree of shoot formation.

4. DIRECT REGENERATION

When relatively large pieces of intact plants are transferred to nutrient media, new shoots, roots, somatic embryos and even flower initials are often formed without the intermediate growth of callus tissue. Small explants show organogenesis only rarely, although some exceptions have been reported. The part of the original plant from which the explant is taken is important in influencing its morphogenetic potential. Many species give rise to adventitious shoot buds on explants taken from a variety of organs including those derived from petioles, stems, roots, leaves and cotyledons. By contrast, adventitious embryos are directly formed much less commonly. They are produced on the leaves of a few plant species, and more frequently on nucellus tissue, or on pre-formed somatic embryos and on the seedlings derived from somatic embryos. Methods of inducing regeneration directly in explanted tissues are being discovered in an increasing number of plant species and direct shoot regeneration is an important means whereby several different kinds of plants may be propagated. It should be noted that plants originating from adventitious shoot or embryo meristems have an increased incidence of somaclonal variation. Direct root formation from non-root explants is relatively common, e.g. *Brassica* (Kartha *et al.,* 1974); *Petunia* (Rao *et al.,* 1973); *Capsicum* (Gunay and Rao, 1978) and *Abelmoschus esculentus* (Mangat and Roy, 1986).

4.1. ORIGIN OF ADVENTITIOUS BUDS

Shoot meristems formed directly on explanted tissues are often initiated by the commencement of cell divisions after about 48 hours in nutrient culture. Each preliminary mitosis is rapidly followed by further division, so that new adventitious primordia are formed, each comprised of a number of cells. These primordia appear to arise in a polar manner in morphogenetically competent explants or parts of explants, and often tend to be spaced equidistantly. Sometimes each meristem appears to originate from one cell, but this is not always the case. There are a number of papers, which describe how such meristems can arise from single epidermal cells (Table 10.1): in the cotyledons of conifers, meristems arise in subdermal mesophyll (Sommer *et al.,* 1975; Cheah and Cheng, 1978; Jansson and Bornman, 1981; Flinn *et al.,* 1988).

Table 10.1 Plants in which adventitious shoots may arise from single epidermal cells

Genus	Reference
Achimenes	Broertjes (1972)
Begonia	Doorbenbos & Karper (1975); Mikkelsen & Sink (1978)
Chrysanthemum	Roest & Bokelmann (1975); Roest (1977); Broertjes *et al.,* (1976)
Linum	Murray *et al.,* (1977)
Kalanchoe	Broertjes & Leffring (1972)
Lilium	Broertjes *et al.,* (1976)
Peperomia	Broertjes *et al.,* (1976)
Saintpaulia	Arisumi & Frazier (1968); Broertjes (1972); Grunewaldt (1977)
Streptocarpus	Broertjes (1969)
Nicotiana	De Nettancourt *et al.,* (1971)

During direct organogenesis, adventitious buds may not all be immediately derived from cells of the explant. There is evidence that a single epidermal cell may sometimes give rise to meristematic centres from one of which up to 22 identical shoots may arise (Broertjes *et al.,* 1976).

The single cell origin of shoots can be confirmed by the high incidence of 'solid' mutant plants, which occurs when suitable organs are subjected to mutagenic treatments. Single cells are mutated and each individual cell may have a different type of mutation from its neighbours. If meristems were initiated from several cells, a high proportion of chimeras would appear. Results such as these have led Broertjes and Van Harten (1978) and Broertjes and Keen (1980) to suggest that directly-regenerated adventitious shoot meristems may always be formed from one or a few daughter cells that originate from a single cell. This was confirmed by Nauerby *et al.* (1991). Others though do not share this view.

Thomas *et al.* (1979) cautioned that extensive histological work is necessary to tell whether

adventitious structures arise from single cells. Following such histological studies, a single cell origin for shoots was found in the basal cells of hairs from *Kohleria* (Geier and Sangwan, 1996). Nevertheless, from a study of shoot regeneration from the leaves of *Saintpaulia* chimeras, Norris and Smith (1981) concluded that the initiation of shoot meristems involves the association and the possible inclusion of cells beneath the epidermis. Adventitious bud meristems which formed between twin bulb scales of onion, arose from many cells in sub-epidermal layers (Falavigna *et al.,* 1980. Similarly, shoots on cultured explants of *Nicotiana tabacum* leaves were found to arise indirectly from nodules at the edges of the explant. The nodules were mainly formed by divisions of palisade mesophyll cells around the edge of the explant where wounding had occurred (Attfield and Evans, 1991). Chimeras have been seen also among the products of adventitious shoot regenerants (Preil, 1986; Broertjes and Van Harten, 1987, Nauerby *et al.,* 1990; 1991) so confirming the multicellular origin of shoots in some circumstances. Moreover, it is clear from studies such as those on *P. sativum* transformed with the GUS gene (Madsen, 1994) that the chimeric shoots are likely to be formed from at least one transformed and one untransformed cell.

In contrast, Attfield and Evans (1991) found that roots in the *Nicotiana tabacum* leaf explants were produced directly from the bundle sheath and vein parenchyma, but the number of cells which participated in their origin was not ascertained. That adventitious root formation is also likely to be multicellular in origin can be seen on rooting from shoot segments of *Pisum sativum* (Gahan and Doré, unpublished data) and from shoot explants of *Malus domestica* var. Jork 9 (Auderset *et al.,* 1994). Roots are seen to arise from parenchymal cells adjacent to, and between the existing vascular tissue. There appears to be a direct influence on these cells arising from the existing vascular tissue and it is possible that IAA or other plant growth regulators diffuse to the adjacent cells and initiate vascularisation and rooting. The procambial tissue is frequently identifiable at the same time as the cells of the initiated root apex. The influence of the existing vascular tissues can be seen on culturing root segments when the cortical parenchymal cells adjacent to the xylem poles in e.g. *Pisum sativum*, will form tracheids prior to the differentiation of the remaining parenchymal cells (Rana and Gahan, 1982).

4.1.1. Meristemoids.

At an early stage of regeneration, a spherical mass of small meristematic cells is formed. The cells are filled with densely staining cytoplasm and contain large nuclei. Such specialised cell groups were called meristemoids by Torrey (1966). Other workers have taken up the term, although the stage at which meristematic cells come to constitute a meristemoid, or the ultimate size of these structures, has not been precisely defined. The word promeristemoid has been used to indicate small organised groups of meristematic cells, less complicated than meristemoids, which are the sites from which organs, or meristemoids, are formed (Villalobos *et al.,* 1984; Flinn *et al.,* 1988).

Meristemoids may be comprised, at least initially, of undetermined cells. Thus, meristematic cell clusters consisting of 6-7 cells formed from pericycle cells adjacent to protoxylem within *Convolvulus* roots, at this stage were considered by Bonnett and Torrey (1966) to be capable of developing into either roots or shoots. Meristemoids formed in other plants have also been considered to be undetermined and capable of developing into either roots or shoots (Thorpe, 1978, 1980; Hicks, 1980), but this interpretation may not be correct. Certainly in some plants, groups of 4-6 meristematic 'proembryoid' cells can become determined towards the formation of a shoot primordium after only 4-7 days of the explant being placed in culture (Flinn *et al.,* 1988).

4.1.2. Predetermination.

According to one hypothesis advanced by Sharp *et al.* (1980); Evans *et al.* (1981) and Sharp and Evans (1982), direct embryogenesis occurs *in vitro* only from cells within the explant, which are predisposed (predetermined) to become embryo-producing before their transfer to culture: nutrient media and other *in vitro* conditions serve only to enhance the process. Such cells are said to be pre-embryogenically determined (PEDCs). An explant will normally contain several types of tissue, not all of which may have this capacity. As an alternative hypothesis, Hepher *et al.* (1988) have suggested that the embryogenic potential of tissues, is related to their active role in nutrient transport: they say that many embryogenic tissues (e.g. nucellus, scutellum, synergids, the abaxial epidermis of soybean cotyledons) have this capacity. These two considerations may not be mutually exclusive since the metabolism of nutrient transporting cells may result in their metabolic status being programmed in a way which is in concert with the predisposition to form embryos.

The parts of a whole plant, which are capable of supporting direct embryogenesis, can give rise, *in vitro*, to tissue of similar appearance to callus, but which has an immediate capacity for embryo regeneration. This tissue can sometimes also be used to initiate suspension cultures with a similar morphogenetic potential (Smith and Krikorian, 1988, 1989). Although such cultures are described in the section on indirect embryogenesis that follows, they probably represent an extended form of direct embryogenesis.

5. INDIRECT REGENERATION

5.1. SEPARATE ROOT AND SHOOT FORMATION

Media and growth regulators that favour rapid cell proliferation and the formation of callus from an explant, are not usually conducive to the initiation of the adventitious meristems, which give rise to roots or shoots. However, in cultures of some plants, organogenesis becomes inevitable when callus is maintained on a medium for a prolonged period, but may be prevented if the tissue is subcultured. In other cases, unorganised callus initiated on one medium needs to be transferred to another of a different composition, with different combinations of growth regulators (a regeneration medium), for shoot initiation to occur .

Organs are formed in callus tissues in a fashion comparable to that observed directly within explanted tissues. Cells from which organs are formed, have arisen from the reprogramming of dedifferentiated cells or, more commonly, have retained a commitment to regeneration which existed in the explanted tissues e.g. shoots from nodal explants of *Pisum sativum* (Nauerby *et al.,* 1990; 1991). This may explain why highly organogenetic cultures are most frequently obtained from young actively dividing parts of plants in which the cells are cycling and so are able to receive signals during G1. Sometimes it appears that meristemoids may have a different origin to other cells of the callus. Callus cultures derived from sections of sweet potato tubers developed meristematic centres of this kind from the cells of anomalous cambia within the tissue of the explant (Hwang *et al.,* 1981). Here, it is possible that the callus has developed from cells not competent for organogenesis, which are adjacent to the competent cells of the anomalous cambia.

The position of meristematic centres may have some relation to the organisation of the explant in newly-initiated callus; but after some while, although roots and shoots do develop within the interior of callus, they commonly occur superficially. This may be related, in part, to the availability of nutrients and to any nutrient gradients set up within the callus. In some plants (e.g. *Convolvulus)* the two different kinds of organ have been shown to arise in different parts of the tissue, roots from cells in the upper part of the callus and shoots from those in contact with the medium (Christianson and Warnick, 1985). Again, it is not clear whether meristemoids are always formed with the capacity to give rise to one specific type of organ, i.e. whether the conditions, which lead to their formation also decide the type of organ that will be produced, or whether meristemoid formation and organ development are separately and sequentially decided. One possible factor for rhizogenesis may relate to the ability to form vascular tissue at an early stage of development. This is supported by quantitative cytochemical analysis of competent to root (M27) and recalcitrant calluses (Cox) of apple. The competent calluses showed the presence of early markers in the parenchymal cells for the formation of vascular tissue whilst the recalcitrant calluses did not (McGarry, 1988; Gahan *et al.,* 1996)

Sometimes adventitious shoots are thought to have arisen by caulogenesis, when in reality they have developed from somatic embryos. Pro-embryos cultured in a medium containing inappropriate growth regulators, produce shoots lacking roots (Sidavas *et al.,* 1990).

5.2. OCCURRENCE OF EMBRYOGENESIS

In the past, somatic embryos have generally been obtained unreliably in tissue culture and their formation has been much less common than the separate formation of roots or shoots. Somatic embryo formation was at first induced in relatively few plant species (Tisserat *et al.,* 1979), but its incidence is steadily being increased as controlling factors become better understood (Williams and Maheswaran, 1986). In many species, indirect embryogenesis was originally noted only fortuitously, but may have occurred more frequently than was suspected. For example, when organogenesis occurred in clover callus cultures, Phillips and Collins (1980) were only able to deduce that somatic embryos had been formed. This was because the first and second leaves on shoots were cotyledon-like and unifoliate respectively: if separate adventitious shoot formation had taken place, all leaves would have

been trifoliate. In other species a difference in leaf shape might be less easily detected.

5.3. DIRECT SOMATIC EMBRYOGENESIS.

Direct embryogenesis, that is to say, the formation of somatic embryos on explanted tissues without any intervening callus phase, is observed much more frequently *in vitro* than is the formation of adventitious embryos *in vivo*. Reports of direct embryogenesis *in vitro* have mainly described its occurrence on gametophyte (the gamete-producing generation) tissues, sporophytic (normal plant) tissues closely associated with the gametophyte, or tissues which have recently arisen in consequence of the fertilisation of gametes. Explants from which direct embryogenesis is most likely to occur include pollen microspores within the anther and tissues of all or part of the ovary [including the ovary wall (or carpels) Smith and Krikorian (1988)], ovule (especially the nucellus), zygotic embryo or young seedling. Physiologically, these explants are all very juvenile. Instances of direct embryo formation are discussed in Chapter 2.

5.4. INDIRECT SOMATIC EMBRYOGENESIS

Somatic embryos can also be produced indirectly in otherwise unorganised cell and tissue cultures. True indirect embryogenesis requires the differentiated cells of an explant to be induced to divide as undifferentiated callus and then for certain cells to be become committed or redetermined on an embryogenic pathway. In the terminology of Sharp *et al.* (1980), cells derived from previously differentiated tissue, which have been newly induced to embryogenic determination are called IEDCs (induced embryogenic determined cells) (c.f. PEDCs in Section 4.1.2.).

5.5. CELLULAR ORIGIN OF SOMATIC EMBRYOS

Because embryogenesis is often difficult to observe, there has been some controversy over its origin in unorganised cultures, but the developmental anatomy (ontogeny) of embryos has been studied in detail in several different species, e.g. *Daucus carota* (McWilliam *et al.,* 1974; Nomura and Komamine, 1985), *Ranunculus scleratus* (Konar *et al.,* 1972) and *Pennisetum americanum* (Vasil and Vasil, 1982).

Embryos can be distinguished from adventitious shoots because they are bipolar, having both a shoot and a root pole, a shoot axis and cotyledons (or a coleoptile and a scutellum in monocotyledons) of a similar venation to that in zygotic embryos. They also have no vascular connections with the underlying parental tissue. This is not as in axillary or adventitious buds which can induce the formation of procambial conducting strands in maternal tissue. In whole plants, these strands establish a connection between the young shoot and the vascular system of the mother plant.

Unlike the meristems that give rise to separate root or shoot initials (which may also be produced internally), somatic embryos nearly always arise superficially on callus and are detached easily from the surrounding cells. They may float free in suspension cultures from the globular stage onwards. It is generally agreed that somatic embryos are formed from cells that are characteristically meristematic and therefore unlike the usual vacuolated parenchymatous cells found in callus and suspension cultures: they have dense cytoplasm and large nuclei and contain many starch grains. However, it is not clear that these cells, and hence somatic embryos, always originate from single determined cells.

5.6. SINGLE CELL ORIGIN

A plant embryo, whether it be zygotic, somatic, directly- or indirectly-initiated, has been defined as a new individual arising from a single cell and having no vascular connection with the maternal tissues (Haccius, 1978). However, the developing embryos *in vivo* e.g. *Pisum sativum*, can be seen to be influenced by the maternal vascular tissue with which there is an early link *in vivo* (P.B. Gahan & M.A. Rana unpublished). It has been clearly shown that somatic embryos can arise directly from a single cell in explanted tissue (Dubois *et al.,* 1990; Alizedah and Mantell, 1991); in both callus and suspension cultures (e.g. in carrot; Backs-Husemann and Reinert, 1970; Nomura and Komamine, 1986: in pear; Mehra and Jaidka, 1985: in fennel; Miura and Tabata, 1986, and in rice; Jones and Rost, 1989); from protoplasts (Miura and Tabata, 1986); and from mechanically-isolated cells (Uragami *et al.,* 1990). Tracking single embryogenic cells showed also the very small number of cells capable of forming embryos (Somleva *et al.,* 2000).

A change in the regular plane of cell division is one of the first indications of the formation of an embryogenetic tissue (Hepher *et al.,* 1988). Where embryos originate from a single cell, an unequal periclinal division results in one small cytoplasmically-rich cell (the terminal cell) and another large vacuolated cell (the basal cell). In some

cultures, the basal cell does not divide further, but in normal circumstances it can be seen to be the source of a suspensor (Fig. 10.6).

5.7. POLYEMBRYONY.

Somatic embryos can often be seen to arise, not from single cells, but from globular clumps of tissue. Haccius (1978) suggested that such pro-embyonal cell complexes still have their origin in single embryogenically-dedicated cells and that formation of embryos from the globular tissue was the equivalent of the cleavage polyembryony which sometimes occurs *in vivo* (i.e. the division of the zygote or pro-embryo into two or more units). In *Dactylis glomerata* it has been proposed that pro-embryonal cell complexes or abnormal embryos are produced instead of normal somatic embryos, if the division of single embryogenically-determined cells proceeds in an anticlinal or random fashion (Trigiano *et al.,* 1989; Fig 10.6). Somatic embryos with both uniseriate and multiseriate suspensors were produced later from pro-embryonal cell masses by cleavage polyembryony.

The emergence of embryos from a globular embryogenic tissue of *Picea* was called polyembryogenesis by Gupta and Durzan (1986; 1987: Durzan and Gupta, 1987) because of its analogy to the cleavage polyembryony which occurs during zygotic embryogenesis in the Pinaceae. In some genera of this family, a group of cells which would usually be expected to form a single embryo, divides (cleaves) into files of cells each of which begins to form an embryo. The genetically identical embryos compete and usually only one survives (Fig 10.7). The very long suspensors produced during zygotic embryogenesis in the Pinaceae are also observed during somatic embryogenesis in this family, so that embryogenic tissue *in vitro* has been called embryonal suspensor masses (Gupta *et al.,* 1988). A review of the terminology in zygotic and somatic embryogenesis in conifers is given by Tautorus *et al.* (1991) (See Chapter 9).

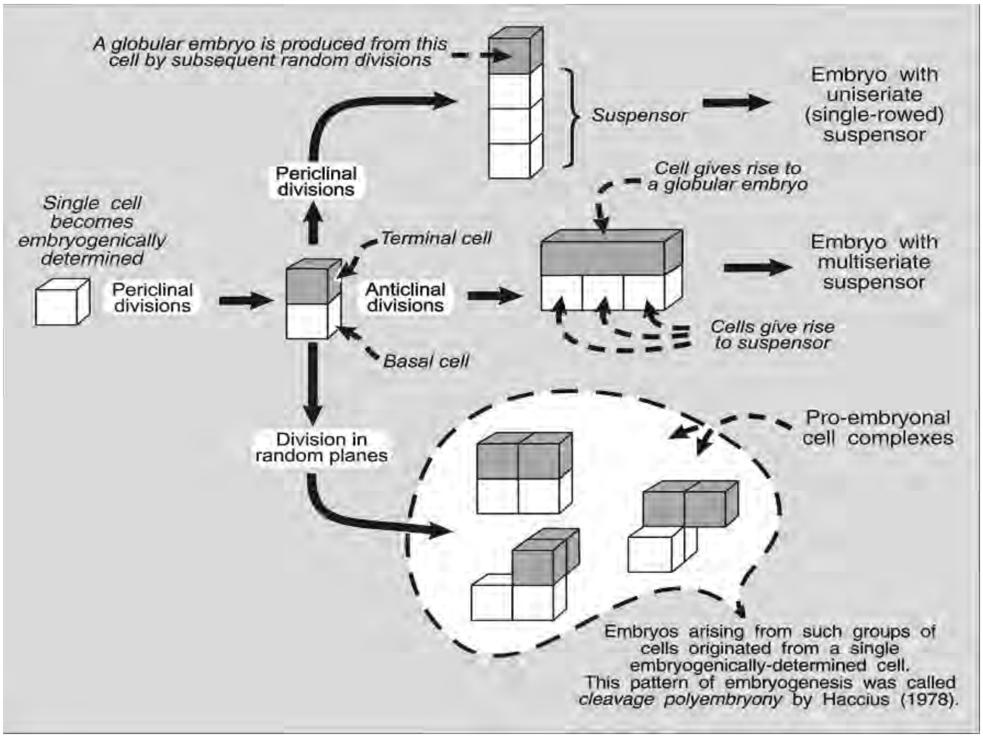

Fig. 10.6 Proembryonal cell complexes result from abnormal planes of cell division during embryo formation from single embryogenic cells [after Trigiano *et al.,* 1989].

Clumps of embryogenic tissue may arise in other ways. In rice it has been noted that many more embryos are initiated from single cells of the scutellum of zygotic embryo explants, than complete their development. Competition for resources (such as occurs during the maturation of one out of several embryos during cleavage polyembryony in gymnosperms) leads to many embryos proliferating, from the globular stage onwards, as parenchymatous 'callus' tissue. Clusters of embryos and related embryogenic callus tissue may form an epithelium similar to that of the scutellum, from which new somatic embryos may be originated (Jones and Rost, 1989). Globular cell clumps (pro-embryonal cell complexes) have also been observed to give rise to somatic embryos from single cells in both callus and suspension cultures of other species (*e.g.*, by Button *et al.,* 1974; Kohlenbach, 1977).

5.8. MULTICELLULAR ORIGIN.

Several authors have maintained that embryos can have a multicellular origin. Maheswaran and Williams (1985) concluded that the initiation of somatic embryos in *Trifolium repens* was predominantly multicellular. The first sign of embryogenesis in immature zygotic embryos was a change in the normal plane of cell division. This was followed by the formation of a file of cells that developed into a meristem. Somatic embryos could arise from single meristematic cells, but they were usually produced by multicellular budding. Proliferation of the meristem resulted in a distinctive globular tissue.

A multicellular origin for somatic embryos by folding or budding of embryogenic tissue has also been recognised in other plants (e.g. *Sorghum bicolor* - Wernicke *et al.,* 1982). Williams and Maheswaran (1986) suggested that it may occur when neighbouring cells are in the same stage of embryogenic induction: a group of adjacent cells may then associate and act conjointly in the process of embryo formation. The presence of broad suspensors on somatic embryos is thought to be indicative of a multicellular origin (Halperin, 1966; Armstrong and Phillips, 1988).

5.9. RATE OF EMBRYOGENESIS

Some callus cultures may produce somatic embryos slowly and so contain only a small number at any given time. The appearance of isolated somatic embryos in coffee calluses was called 'low frequency somatic embryogenesis' by Sondahl and Sharp (1977a, b, c, 1979) and Sondahl *et al.* (1979a,b). Embryogenic callus that comes to contain proliferating pro-embryonal tissue is usually different in appearance to non-embryogenic tissue: it can give rise to large numbers of somatic embryos. Such callus sometimes arises directly upon an explant, and on other occasions from non-embryogenic tissue, or tissue giving rise to a small number of embryos, after a period of culture. Appearance of a recognisably different globular embryogenic tissue from a high proportion of coffee calluses previously disposed to low frequency embryogenesis was termed 'high frequency somatic embryogenesis' by Sondahl *et al.* (loc. cit.).

5.10. PSEUDOBULBILS AND PROTOCORMS

Some calluses can be highly embryogenic and may consist entirely of globular cell masses from which new ones continually arise by budding. In other cultures, clumps of embryogenic meristematic cells form spherical nodules on the surface of callus, or cellular aggregates in suspension cultures. Globular bodies tend to reach a larger size under conditions that are not fully conducive to embryogenesis and are then sometimes called pseudobulbils (La Rue, 1954; Ranga Swamy, 1958; 1961).

Similar globular bodies, which frequently form on callus cultures of orchids, are not pseudobulbils, but protocorm-like bodies. These are the *in vitro* equivalent of the protocorms formed during the germination of orchid seeds..

5.11. PROLIFERATION OF PRE-EMBRYOGENICALLY DETERMINED CELLS

As explained in the section on Direct Embryogenesis, some explants (such as the nucellus of poly-embryonic *Citrus* species - see review by Rangaswamy, 1982), capable of direct embryogenesis, are invariably able to give rise to an embryogenic tissue or callus. Usually it occurs together with a simultaneous outgrowth of truly unorganised tissue. In grapevines, a white tissue (which the authors said did not look like callus because it consisted of a mass of tiny individually organised units) appeared from the shoot apices and cotyledon regions of seed embryos and was accompanied by a creamy yellow unorganised friable callus. Immature polyembryonic zygotic embryos of conifers can be stimulated *in vitro* to proliferate a white embryonal tissue (an embryonal suspensor mass) from the suspensor region at the radical end, in conjunction with green non-embryogenic callus from other regions.

In cases such as those instanced above, indirect embryogenesis might be said to have occurred because somatic embryos did not appear on the

primary explant. On the other hand, the embryogenic tissues appear to originate directly from pre-embryogenically determined cells and are not undifferentiated. The embryonal suspensor masses of *Picea* can be converted into somatic embryos through a change of medium composition (e.g. the addition of boron (Zoglauer *et al.,* 1998) and growth regulators (Krogstrup *et al.,* 1988). The distinction between direct and indirect embryogenesis is clearly blurred, especially as in many reports of embryogenesis, it is not obvious as to whether or not the explant was embryogenically determined.

The embryogenic determination of tissues from different sites on a single plant, or from similar explants derived from different genotypes, clearly varies. Culturing explants on a simple medium without growth regulators occasionally produces highly embryogenic callus. However, the induction of embryogenesis usually requires explanted tissues to be placed on a medium containing an auxin. Growth regulators of this kind stimulate cell division and cause cells to become committed to embryo formation. In callus that has been grown for some while in an unorganised state without the induction of regeneration, the dedifferentiated cells require to be completely redetermined into an embryogenic condition before they can give rise to somatic embryos. Auxins are once again primarily responsible for bringing about this altered commitment.

5.12. STAGES OF EMBRYO DEVELOPMENT

The close structural similarity between somatic and zygotic embryos has naturally created much interest. Although within somatic embryos there is not an orderly pattern of cell division planes (once thought to be important for the eventual designation of cell function in zygotic embryos), variation in these segmentation patterns probably only reflects the different environment in which the cultured cells were contained and is, in any case, also known to occur sometimes during zygotic embryo growth (Street and Withers, 1974). As additionally, the later stages of development of zygotic and somatic embryos are very similar, it may be supposed that the processes of zygotic and somatic embryogenesis are essentially the same. The various stages in the development of somatic embryos in dicotyledons are shown diagrammatically in Fig. 10.8

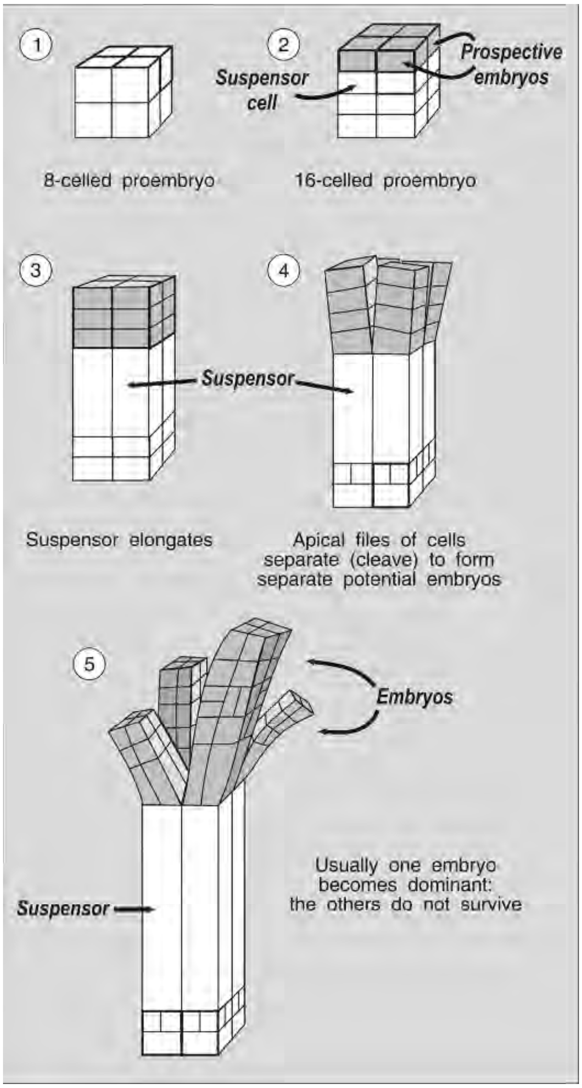

Fig. 10.7 A diagrammatic representation of zygotic cleavage embryogenesis in *Pinus*.

They are usually described as follows

- pro-embyros: small clusters of meristematic cells from which somatic embryos will arise,
- globular stage: larger groups of cells not yet having a definite embryo-like shape,
- heart stage: a characteristic three-lobed stage where cotyledonary initials are separated from the root pole,
- torpedo stage: an elongated form of the heart-shaped embryo,
- plantlet: discernable small 'seedling' with primary root and shoot.

The stages in monocotyledons are somewhat different; at the globular stage, somatic embryos are easily confused with spherical shoot meristems and it can be difficult to be sure that embryogenesis has taken place. However, the embryos of monocotyledons give rise to shoots from discrete globular bodies, which, as they grow, develop structures resembling a scutellum and coleoptile (Fig. 10.8); adventitious shoot buds form a clump of leaves. Somatic embryos also typically develop root and shoot poles and are discontinuous with the underlying callus.

Pro-embryos of both dicotyledons and monocotyledons typically have a number of cells at the root pole end, which are equivalent to the suspensor of zygotic embryos; these cells may not be apparent in embryos which arise by multicellular budding, where the somatic embryo can be closely associated with the parent tissue.

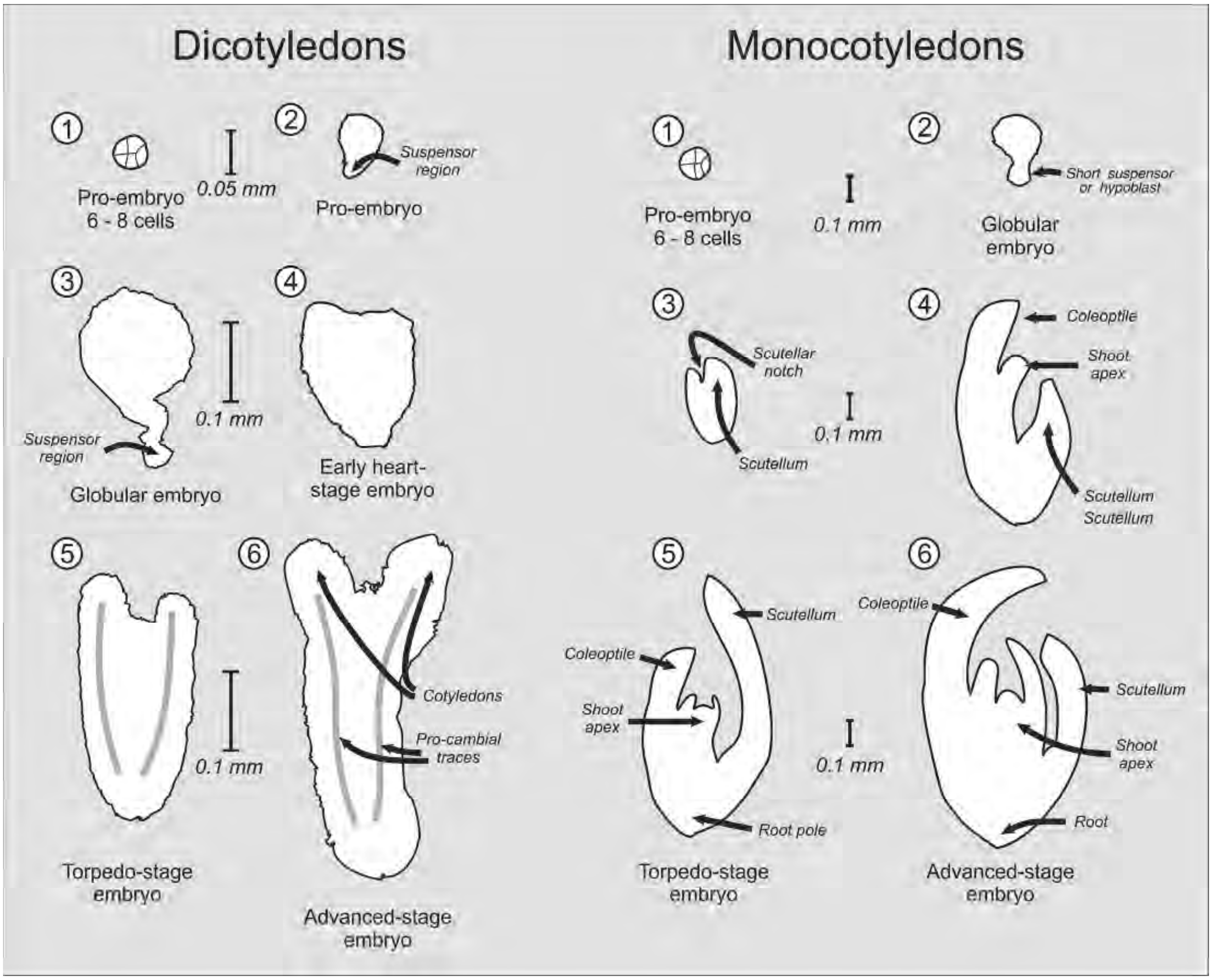

Fig. 10.8 Stages of somatic embryogenesis in dicotyledons (*left*) and in monocotyledons (*right*).

Fujimura and Komamine (1980) found that in carrot, very different rates of cell division occur at different stages of somatic embryo formation. A marked increase in rate occurs after the pro-embryo stage and precedes the formation of globular embryos. In maize, the start of both zygotic and somatic embryogenesis can be detected through changes in isoenzymes (Everett *et al.,* 1985). Although there may be only a small change in the total population of proteins during embryogenesis (Sung *et al.,* 1988), somatic embryos do contain some specific proteins not found in other tissues (Hahne *et al.,* 1988). The amounts and nature of these may change as growth proceeds (Zee and Wu, 1979): storage proteins accumulate which are not normally found in somatic tissues (Crouch, 1982; Stuart and

Nelsen, 1988). Electrical currents have been found to move through globular bodies of embryogenic callus, pro-embryos and early stage embryos demonstrating that a distinctive polarity exists in these structures from a very early stage (Brawley *et al.,* 1984).

5.13. GERMINATION.

Somatic embryos which have been suitably matured can be germinated *in vitro* and sometimes *ex vitro* to produce normal seedlings. Very large numbers of somatic embryos can be produced in some cultures, but normally only a small percentage of these ultimately gives rise to plants. The proportion which does, has been termed the conversion frequency by Redenbaugh *et al.* (1986, 1987)

6. THE EFFECTS OF GENOTYPE

The growth of cultured tissues or organs, and regeneration *in vitro*, are very much influenced by the genotype. There are numerous examples in the literature in which the results obtained during tissue culture have varied from one variety of plant to another, and it is probably true to say that effects of genotype impose one of the greatest constraints to the tissue culture and micropropagation of plants. In some instances, observed responses have been related by identifiable genetic factors, viz.: nuclear genes (Bullock *et al.,* 1982; Foroughi-Wehr *et al.,* 1982; Lazar *et al.,* 1984; Rhodes *et al.,* 1986; Szakcs *et al.,* 1988; and Mathias and Fukui, 1986; Lall *et al.,* 2004), cytoplasmic genes Mathias *et al.,* 1986; Narasimhulu *et al.,* 1988; Wan *et al.,* 1988); and gene interactions (Keyes and Bingham, 1979; Wan *et al.,* 1988).

Nuclear genes governing regeneration have been thought to have a high general ability to interact (tomato - Frankenberger and Tigchelaar, 1980); to be recessive (tomato - Frankenberger *et al.,* 1981); to be dominant, or partially dominant (alfalfa - Reisch and Bingham, 1980: *Zea mays* - Rhodes *et al.,* 1986); or to be additive (Brassica - Buiatti *et al.,* 1974a, b). Ploidy is found to have an effect in some genera: dihaploid lines of *Solanum tuberosum* are more likely to produce adventitious shoots from callus than are their tetraploid parents (Fish and Jones, 1988).

Many genotype-dependent effects are caused by interactions between the plant's genotype and the cultural environment. Variety A may undergo regeneration in response to growth regulators of one kind, while variety B is unresponsive until the concentration of the regulators is changed and/or different regulatory compounds are used. Other differential responses can be due to plants of varying genotypes requiring specific media or environmental conditions.

Because of genotypic specificity, media and the cultural environment often need to be varied from one genus or species of plant to another. Although general methodologies can be established for plant tissue culture, even closely related varieties of plants can differ in their cultural requirements. The best method of micro-propagating a new plant must usually be determined by experiment. It has been particularly difficult to obtain regulated regeneration of many plants within the families Fabaceae and Poaceae.

6.1 PREDOMINANTLY GENOTYPIC EFFECTS

If interaction with the environment were the only cause of genotype specificity, it should be possible to culture all plants of a similar kind with equal success once the specific conditions for each had been ascertained. Unfortunately, the behaviour of plant tissues *in vitro* in processes such as callus formation and growth and regeneration often seem to be under an over-riding genetic control, with other factors exerting only a minor effect. Thus in wheat, some genotypes give rise to plants from tissues cultured on several different media, while little success is obtained with other varieties on any of these choices (Lazar *et al.,* 1983). Similarly in *Medicago* more plants were produced from somatic embryos in highly regenerative cultivars than from those with a low capacity for regeneration, regardless of the media employed (Brown and Atanassov, 1985). It appears that the inability to induce regeneration in some genotypes is due rather to our incomplete knowledge than to an over-riding genetic constraint.

Plant genotype has a sufficiently marked effect on the behaviour of cultured plant tissues for it to be possible to breed plants for improvements in all aspects of *in vitro* performance (see below). Some of the more obvious ways in which growth and regeneration have been found to be influenced by genotype are discussed below.

6.1.1. Culture initiation

Surprisingly, in some genera, the rate at which explants survive in culture can depend on apparently minor differences in the genotype of mother plants. Thus, there were large differences in the capacity of shoot tip explants from different mature *Eucalyptus marginata* trees to grow *in vitro* after the necessary sterilisation procedures (McComb and Bennett, 1982). The survival of *Dahlia* meristem tips in culture varied even between closely related plant varieties (Wang *et al.,* 1988). Hammerschlag (1982) found a big difference in the viability of newly explanted shoot tips derived from different peach cultivars, whilst Roca *et al.* (1978) recorded that varieties of potato and their hybrids differed more in their behaviour to shoot tip initiation than in shoot proliferation at Stage II.

6.1.2. Callus Initiation and growth

Many reports illustrate how the capacity of explanted tissues to form callus and the subsequent growth rate of callus cultures, can both be variety-dependent. Results from the following plants provide some examples: *Brassica* (Baroncelli *et al.,* 1974), *Helianthus* (Espinasse and Lay, 1989), maize (Shannon and Batey, 1973; Green and Phillips, 1975), oats (Cummings *et al.,* 1976), *Pelargonium* (Jelaska and Jelencic, 1980), *Petunia* (Izhar and Power, 1977), potato (Simon and Peloquin, 1977), rice (Abe and Sasahara, 1982), sainfoin (Arcioni and Mariotti, 1983), tobacco (Venketeswaran and Mahlberg, 1962; Cheng and Smith, 1973; Keyes *et al.,* 1981), and wheat (Shimada and Makino, 1975; Sears and Deckard, 1982). The effect of genotype is also seen in differences in the texture and colour (Taira *et al.,* 1977), and morphogenetic capacity of callus from closely related varieties of plants.

The results obtained in any particular test depend on the medium, the growth regulators used, and other environmental factors. Under the conditions employed by Gresshoff and Doy (1972), callus cultures could be established from only three out of eighteen *Arabidopsis thaliana* genotypes, and one out of forty-three barley lines. Bayliss and Dunn (1979), however, could plot a normal distribution of the amount of callus produced from the seed embryos of each of 45 randomly chosen barley varieties on MS medium containing 1 mg/l 2,4-D.

Baroncelli *et al.* (1978) found that the genetic control of wheat callus growth was different in root and nodal explants, and a somewhat similar result was obtained in rice by Mikami and Kinoshita (1988). Although vigorously-growing callus was formed on the tissues of only some varieties, the growth response of seed, coleoptile and radicle explants was broadly related. However, the ability of the varieties to form callus from anthers was not correlated with the results obtained from the other explants. The authors suggested that their results might have been due to there being different genetic determinants of callus formation according to the physiological condition of the explants or according to their ploidy.

6.1.3. Direct regeneration

Adventitious shoots. Plants vary considerably in their ability to produce adventitious shoots directly on tissue explants. In general, success *in vitro* closely parallels the ease with which adventitious shoots may be regenerated without resort to aseptic culture, except that under controlled and disease-free conditions, the morphogenetic potential of the species is magnified, and shoots are produced more freely than they otherwise would be. Thus, those genera, which produce plantlets readily from severed leaves placed in soil or compost in a greenhouse (e.g. *Begonia*, *Peperomia*, *Saintpaulia*, and *Streptocarpus*) also give rise to large numbers of adventitious shoots directly from leaf or petiole segments when these are cultured on an appropriate medium. In some other kinds of plants, aseptic culture can be used to induce direct shoot regeneration that otherwise is seldom observed. *Chrysanthemum morifolium*, for instance, can give rise to adventitious shoots *in vitro* from explants of small leaves, petioles, stems, flower pedicels or young flower buds. Direct shoot regeneration commonly occurs on the explanted tissues of dicotyledonous species, but in monocotyledons is only normally observed in species which produce fleshy storage leaves.

Species that produce bulbs seem to be universally capable of producing adventitious shoots from the basal part of explanted bulb scale leaves. According to Broertjes *et al.* (1968), only monocotyledons of the families Amaryllidaceae, Araceae, Liliaceae and Taccaceae are capable of forming adventitious shoots on leaf tissue. Many of these plants can also produce buds on floral stems (scapes).

Despite these very broad differences between species and genera, the capacity for direct regeneration on any given medium can also be found to vary between closely related plant varieties. Mutant lines of *Arabidopsis thaliana* had a lower shoot- and root-forming capacity than the normal (parent) line, but morphogenetic ability was fully restored in their hybrids (Nobuharu, 1980; Goto,

1981). This is an illustration of complementation, each of the pair of genomes present in the hybrid supplying one or more products, which were either absent or impaired by the mutation in the other. Sometimes the hybrids would show heterosis (hybrid vigour) by exceeding the organogenetic capability of the normal lines.

The shoot forming capacities of two pure lines of tomato ('Apedice' and 'Porphyre') and their reciprocal hybrids, were found by Bigot *et al.* (1977) to vary according to the derivation of explants and the growth regulators used. Shoots were initiated directly on the explanted tissue and/or on callus produced from superficial tissues. 'Porphyre' always produced more shoots from hypocotyl explants than 'Apedice'. This ability was maternally transmitted to one of the hybrids and, with one particular combination of auxin and cytokinin, hybrids produced significantly more shoots than did the parents (a further example of heterosis). The relative regenerative capacity of the genotypes studied was not the same when cotyledon or leaf explants were cultured.

Root formation. Whether roots or shoots can be induced to form in callus cultures can frequently be controlled by addition of growth regulators, but in some cultures even closely related genotypes may have different propensities (Kim *et al.,* 1988). Similarly, the facility with which adventitious roots can be induced to form directly on shoots grown *in vitro*, varies widely, sometimes even amongst varieties of the same species.

Embryogenesis. The ability of closely related plants to produce somatic embryos directly on explants is also under genetic control and differences between varieties are often found. Stamp and Meredith (1988) obtained somatic embryos on the zygotic embryos of four cultivars of *Vitis vinifera*, but could not induce them to form on the cultivar 'Pinot Noir'. The direct formation of somatic embryos on apple leaf segments was genotype-dependent (Welander, 1988).

Indirect regeneration. Genotypic effects are influential in the regulation of adventitious organ formation from callus tissues. Separate root and shoot formation, or embryogenesis, is readily induced in plants of certain families or genera, and is obtained with difficulty in others. Differences in the morphogenetic capacity of callus from different species or even varieties of plant are also commonly found e.g. *Peperomia* (Klimaszewska, 1979), *Nicotiana* (Bourgin *et al.,* 1979), *Triticum* (Gosch-Wackerle *et al.,* 1979).

To the extent that indirect regeneration is impossible without prior callus formation, callus growth and regeneration are connected. The rate of callus proliferation from genetically similar plants may not, however, always be a guide to the morphogenetic capacity of the callus. In other cases morphogenetic competence and callus production seem to be influenced by the same genes. In cauliflower, the weight of callus produced on leaf explants of inbred lines was related to the number of shoots formed directly on petal explants of the same varieties (Baroncelli *et al.,* 1973).

Intervarietal differences in the morphogenetic capacities of callus tissues are also commonly found. The rate of adventitious shoot formation can vary between lines within a species e.g. *Glycine canescens* (Hammatt *et al.,* 1987), and the facility with which embryogenesis can be induced may vary between cultivars e.g. *Solanum melongena* (Matsuoka and Hinata, 1979); alfalfa (Brown and Atanassov, 1985). In many experiments to study plant regeneration, callus of different plant varieties is induced to form on a single medium, but frequently when the calluses are plated onto a common regeneration medium, each has a different organogenetic potential (e.g. Barg and Umiel, 1977; Margara, 1977; Ohki *et al.,* 1978). Cummings *et al.* (1976) obtained plantlet formation from callus of 16 out of 23 varieties of oats, and Wehner and Locy (1981), shoot regeneration from only 28 out of 85 cucumber lines, although callus cultures were produced from explants in every case.

7. GENETIC CONTROL

The genetic control of growth and regeneration *in vitro* can result from either a primary or secondary effect of gene action. Thus, certain kinds of morphogenetic responses in tissue cultures are seen to be directly related to the behaviour exhibited by whole plants or parts of plants *in vivo* e.g. morphological features, whereas features of the whole plant which influence other characteristics of *in vitro* culture, e.g. the rate of callus growth, are more difficult to determine. Genetically determined characteristics of the latter kind probably result from only the secondary effect of normal gene function. This represents a spurious pleiotropy in which the main effect of a gene or genes causes other unrelated events to occur.

By correlating both the rate of callus growth and the ability to form adventitious buds with characters of the mature plant, Baroncelli *et al.* (1974) suggested that in inbred lines of cauliflower there might be groups of genes capable of shifting plant development in different directions, their effect in culture being to influence the competing processes of cell proliferation and regeneration. Such genes have been thought most likely to exert their influence by regulating the effective levels of growth substances (Bayliss and Dunn, 1979). Endogenous growth substance levels may vary in different genotypes.

Inbred varieties of several plants have been found to produce callus less well than their hybrid progeny, in which heterosis seems to be manifest (e.g. from explants of maize endosperm, (Tabata and Motoyoshi, 1965); alfalfa, (Keyes and Bingham, 1979); tobacco cotyledons, (Keyes *et al.,* 1981). Heterosis may also be seen in the ability of anthers to form callus (Bullock *et al.,* 1982) or produce haploid embryos. Keyes *et al.* (1981) noted how in several plants, correlations were found between the rate of growth of tissues *in vitro*, and vigour in other agronomic traits. They suggested that characteristics of *in vitro* cultures might be of value for the rapid identification of hybrid combinations that could produce superior plants in the field. In *Petunia hybrida*, however, Hanson and Read (1981) found that although F_1 hybrids generally gave more vigorous plants and callus cultures than their inbred parents, there was no consistent correlation between tissue culture and field performance. Callus from the shoot meristems of the interspecific hybrid between *Trifolium hybridum* and *T. ambiguum* grew more slowly than that of either of the parental species (Rupert *et al.,* 1976).

Some of the characteristics of *in vitro* cultures of closely related genotypes have been subjected to biometrical analyses and in *Brassica oleracea* have been shown to be correlated in a complex fashion with apparently unrelated features of whole plants *in vivo* (Baroncelli *et al.,* 1973, 1974; Buiatti *et al.,* 1974a, b).

Genes governing the capacity for *in vitro* callus growth have been shown to be located in particular portions of wheat chromosomes (Shimada and Makino, 1975; Baroncelli *et al.,* 1978), and genes situated on *Nicotiana glauca* chromosomes were thought to be responsible for the relative rates of callus proliferation of triploid hybrid plants resulting from a *N. glauca* and *N. langsdorffii* cross (Cheng and Smith, 1973). Genetic factors governing callus formation and shoot regeneration in *N. tabacum* were deduced to reside chiefly in the part of its genome derived from *N. sylvestris* (Ogura and Tsuji, 1977).

Embryogenic callus resulted from culturing immature embryos of a *Vigna glabrescens V. radiata* hybrid, but only non-morphogenetic callus could be obtained from embryos of the reciprocal cross (Chen *et al.,* 1990).

Selection. Some *in vitro* characters can have high heritabilities. In inbred cauliflower lines, Baroncelli and co-workers (1973) found values of 0.42 for the number of roots formed per callus, 0.39 for the proportion of petals which gave rise to callus, and 0.47 for the relative rates of callus growth. Selection amongst progeny segregating for these characteristics would clearly have resulted in improvement. Since then it has been clearly established that it is possible to improve upon the results obtained from plant tissues and organs *in vitro* by conventional plant breeding techniques.

Recurrent selection within a species has been effective in increasing the proportion of genotypes capable of regeneration from callus (e.g. from tetraploid alfalfa callus - Bingham *et al.,* 1975; Reisch and Bingham, 1980), and has enabled the selection and release of tetraploid alfalfa cultivars (Regen S, Regen Y and RA-3) having a high capacity for regeneration from tissue cultures. Selection of a diploid clone having this attribute has also been possible (McCoy and Bingham, 1977, 1979). Lines capable of improved regeneration could be selected from the progeny of crosses between *Trifolium* genotypes capable of producing adventitious buds directly from explants (Webb *et al.,* 1987).

8. THE EXPLANT

The kind of explant chosen, its size, age, and the manner in which it is cultured, can all affect whether tissue cultures can be successfully initiated, and whether regeneration can be induced.

8.1. THE AGE OF EXPLANTED TISSUES

The successful culture of plant material *in vitro* is greatly influenced by the age of the tissue or organ which is used as the initial explant. The age of any piece of tissue can be measured in five ways, i.e. by:

- **The age of the organ or whole plant from which it is derived**. This is the period of time (hours, days, months) that has elapsed from the growth of a plant from a bud or embryo, or from the initiation of a particular organ or tissue.
- **Physiological age** in which the time taken to reach a particular stage is influenced by environmental factors e.g. temperature.
- **Ontogenetic age or phase of growth.** In this sense a piece of tissue can be categorised as being 'young' or 'old', according to whether it is derived from a part of a plant in a juvenile or adult phase of growth. The marked effects of 'maturation state' on plant tissue cultures have been discussed in the section on phases of growth at the beginning of this chapter.
- **The degree of differentiation**. In this concept, the youngest parts of a plant are undifferentiated meristematic cells, while differentiated cells of organs, sequentially derived from the meristems, are older. Developmentally, the cells of the apical meristems of most plants have a theoretical capacity to enjoy 'perpetual youth' and to live for ever if the apex of the plant is continually re-rooted *in vivo* or subcultured *in vitro*.
- **Period of culture**, the time span from when the culture or the passage was first commenced. Once a culture has been initiated from explanted material, this fifth age factor becomes operational.

Each of these components of tissue age may influence whether an explant from a stock plant or from a previous *in vitro* culture, can be used to initiate a culture, and may modify the direct or indirect morphogenetic potential of the tissue. Ontogenetic age and the age of the organ appear to be the most important factors, although in practice it is sometimes impossible to decide which of the first four age factors above is decisive. The shoot apex of a seedling is young in all senses, but although the stem apex from a 50 year-old tree is old in the material sense, it is youthful in that it is, essentially, undifferentiated. Nevertheless, although the shoot apex is comprised of actively dividing cells which are morphologically similar, it important to note that some of these cells appear to carry the first phase of the genetic programme for vascularisation as was shown for *Vicia faba* and *Pisum sativum* (Gahan and Bellani, 1984). Moreover, the cells which will give rise to the cortex are likely to already be programmed for this differentiation process. This is clearer in the root meristems where the central core of meristem cells carry the first phase of the genetic programme for the formation of vascular tissues and the endodermis (Gahan, 1981; Rana and Gahan, 1983; Gahan and Rana, 1985; Gahan and Carmignac, 1989).

8.2. ONTOGENETIC AGE

Explants taken from juvenile plant tissue, particularly that of seedlings, are usually highly responsive. This can be a genuine effect of material age, but usually reflects the juvenile state of seedling tissues. The difficulty, which is often experienced in growing and manipulating older tissues can be frustrating, because it is usually desirable to propagate mature plants of proven worth. This is especially so in the propagation of trees.

8.3. AGE OF THE ORGAN OR PLANT

In general, explants taken from newly originated organs are most likely to be capable of growth and organogenesis *in vitro*.

8.3.1. Callus initiation

Growth regulator requirements for callus initiation frequently differ according to the age of the explanted tissue. On a modified Linsmaier and Skoog (1965) medium, maximum growth of callus from stem explants taken from 2 week-old *Pelargonium* plants, occurred in the presence of 5 mg/l NAA + 5 mg/l kinetin; but these concentrations inhibited callus formation from similar explants taken from 2 year-old plants. Optimum callus proliferation from the latter occurred with 0.2 mg/l of both regulators (Hammerschlag and Bottino, 1981). The formation of meristemoids, and eventually shoots and roots, occurred only on the young explants cultured on 0.2 mg/l NAA and 0.2 mg/l kinetin.

8.3.2. Indirect regeneration

In experiments of Takayama and Misawa (1982), most segments derived from young leaves of *Begonia* produced buds and roots, whereas those derived from mature leaves usually died. An effect of age was even detected amongst the leaves taken from micropropagated shoots of *Malus domestica*. With an appropriate combination of growth regulants, adventitious shoots formed on the leaves, but the proportion of explants on which regeneration was observed, and the number of new shoots per explant, was greatest in the 3 or 4 leaves nearest the tip of each shoot (Fasolo *et al.,* 1988). Pierik (1969) and Pierik and Steegmans (1975) found that the rhizogenic potential of *Rhododendron* stem segments

decreased with the age of the shoot from which they were obtained, and in the cultivar which rooted least readily, rooting occurred only on segments of soft young stems.

Young tissues do not invariably provide the most highly morphogenetic explants. Callus formed more readily on various explants from fully-grown plants of *Parthenium hysterophorus* than on young seedlings or cotyledons (Wickham *et al.,* 1980). Similarly, callus growth and shoot development was more rapid on expants of *Solanum laciniatum* cut from mature leaves which had just completed full expansion. By contrast, young unexpanded leaves yielded explants which soon expanded and callused but produced shoots slowly, and really old leaves gave rise to callus and shoots after an even greater time interval. The number of shoots regenerated from the three types of tissue was eventually very similar (Davies and Dale, 1979).

Frequently, the pattern of regeneration changes as a tissue ages and then the effect of polarity (see later) on growth and regeneration may become more pronounced. Tanimoto and Harada (1980) took leaf disks from the second pair of unfolded leaves of 9, 14, 18 and 24 week-old plants of *Perilla frutescens*. Explants from the younger plants formed most buds from primary callus. No shoot initiation occurred in callus derived from 24 week-old plants. Young leaves of *Heloniopsis* showed none of the polarity in bud formation present in older leaves (Kato, 1974), and in *Chamaenerion angustifolium* and *Armoracia rusticana*, organs developed along the undamaged margins of young root segments without callusing of the cut ends (Emery, 1955; Dor, 1955), but callusing of the cut ends leading to organogenesis can occur in old roots (Emery, 1955; Bowes, 1976b). Young leaves of *Echeveria elegans* tended to produce roots sooner than they produced shoots, while in older leaves the reverse occurred (Raju and Mann, 1970).

For some types of cultures it may be necessary to excise explants at a fairly precise age. For example, to obtain embryo growth and germination from ovaries of *Brassica juncea*, it was necessary to culture 5-7 day-old ovaries. Those which were 4 or 9 days old were unsatisfactory (Mohapatra and Bajaj, 1988). Immature embryos (without testa and endosperm) of *Malus domestica* which were 0-4 weeks old produced only callus when cultured, those which were 6.5 to ca. 14 weeks produced multiple shoots, whereas mature embryos (14 weeks) grew normally with a shoot and a root pole (Kouider *et al.,* 1985). Depending on whether callus or shoot regeneration was required, seed-derived tissues of *Pyrus serotina* were best excised at the times shown in Table 10.2.

8.3.3. Explants for shoot cultures.

In temperate plants where shoots are newly initiated each year from resting buds, shoots can be said to become progressively older as the season advances. Explants for shoot tip or meristem culture are usually best dissected from young shoots (Kaul, 1985, 1986), or from buds just before the spring flush (Kurz, 1986). On the other hand, lateral buds most capable of growth or shoot proliferation may not necessarily be those nearest the apex of a shoot. Single node explants of *Vitis rotundifolia* taken from the basal 10 nodes of non-lignified shoots bearing at least 25 nodes, were better than those from the 10 distal nodes (Sudarsono and Goldy, 1991).

Table 10.2 The optimum time for taking explants from *Pyrus serotinia* fruits for callus formation and indirect shoot regeneration [Hiratsuka and Katagiri, 1988]

Explant	Optimum age for sampling (weeks after pollination)	
	For callus formation	For adventitious shoots
Immature seed	7-10	Multiple shoots during 8-10
Endosperm	2-4 only	None
Zygotic embryo	2-3 good 4-7 less good 9-10 good	9-10 (later embryos dormant)

Selection of an explant of the optimum ‘age’ for micropropagation can sometimes be complex. In *Rosa hybrida*, single node explants (1 cm of stem plus a bud) were best taken from the new shoots which grew after bushes were pruned when the shoots were ca. 10 cm and had 6 axillary buds. All the lateral buds were then capable of growing *in vitro*. Much less reliable explants were obtained from shoots which had extended until they bore terminal flower buds. Explants which grew well and produced

vigorous shoots could then only be obtained from middle order buds, but by the time the shoots were over 60 cm in length, growth of cultured buds was erratic (Fig 10.9) (Mederos and Rodrguez Enrquez, 1987). A similar observation was made by Kouadio and Phan (1987) with single node explants from tomato plants at the seventh leaf stage. Buds from nodes 1-3 grew slowly in culture, those from nodes 4 and 5 grew most rapidly, while those from node 6 grew slightly less quickly.

The capacity of meristems in onion flower heads to give rise to shoots, was highest when they were at a primordial stage. Some shoots were produced while pollen meiosis was taking place, but after this the ability of the flower heads to form shoots fell to zero (Dunstan and Short, 1979).

8.4. DEGREE OF DIFFERENTIATION

Explants of young tissues still undergoing cell division generally form callus more readily than those from older parts of a plant, and there is a much greater chance that the callus will be capable of organogenesis. This is particularly the case in the Poaceae, where callus cultures have not been initiated from mature leaves or stems, but only from young leaves or inflorescences, seed embryos or nodes. Callus derived from cotyledons excised from 2-4 week-old seedlings of *Pseudotsuga menziesii* had a greater capacity for caulogenesis than that which originated from older seedlings (Abo El-Nil and Wochok, 1977).

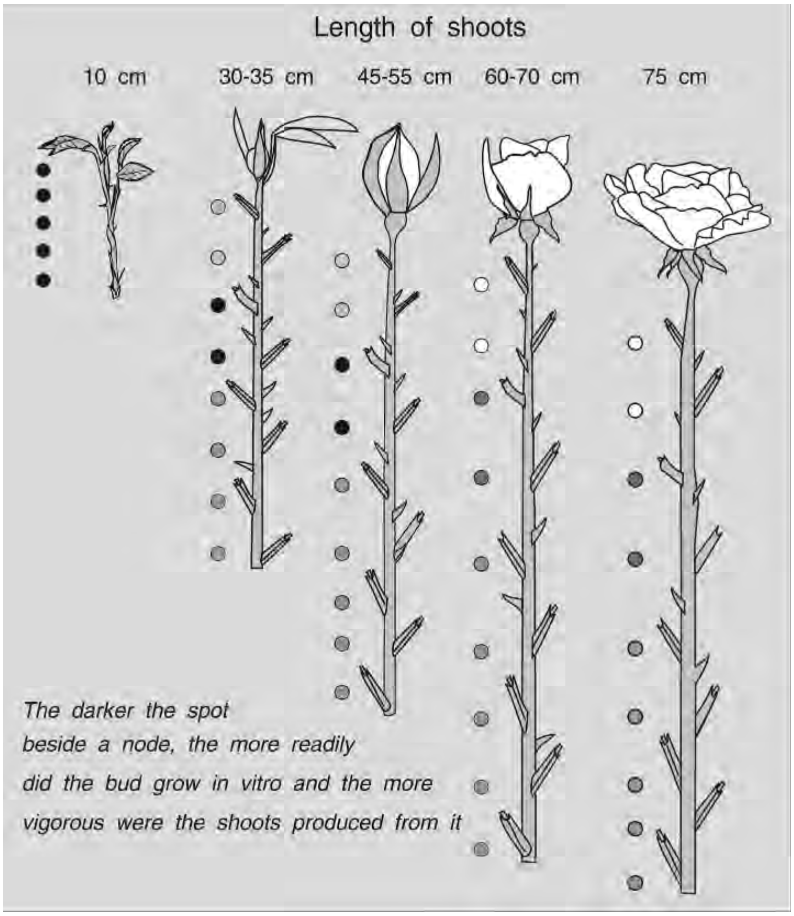

Fig. 10.9 Selection of single node explants of *Rosa hydrida* [after Mederos and Rodriguez Enriquez, 1988].

8.4.1. Zygotic embryos as explants

In a great many species, embryogenic callus can be very reliably obtained from immature zygotic embryos. To improve the yield of embryogenic callus, zygotic embryos of wheat have been classified into the five developmental stages shown in Table 10.3. The highest yields of callus have been found to be produced using embryos at stages II and III (He *et al.,* 1986). Competence of zygotic embryos of *Picea* for somatic embryogenesis has been shown to be limited to a specific stage of development prior to the accumulation of storage proteins (Roberts *et al.,* 1989).

In dicotyledons also, embryogenic callus may only be obtained from zygotic embryos once they have reached a critical size. To obtain embryogenic callus from zygotic embryos of *Castanea sativa* in Spain, it was necessary not to excise the embryos from fruits until the end of August. Those explanted before this time only produced a small callus, or no callus at all (Vieitez *et al.,* 1990). Guerra and Handro (1988) postulated that the induction of direct somatic embryogenesis on *Euterpe edulis* zygotic embryos depended on a subtle interaction between the developmental stage of the explants and the auxin 2,4-D in the medium. Mature embryos of *Iris pumila* gave rise to embryogenic callus once they had been germinated *in vitro*, but immature embryos only produced non-embryogenic callus (Radojevic *et al.,* 1987).

Table 10.3 Stages in the development of wheat embryos [He *et al.,* 1986].

Stage	Days after anthesis	Length mm.	Appearance of scutellum	Characteristics of embryos
I	<13	0.9	Transparent	-
II	12-13	0.9-1.5	Transparent	Visible
III	15-17	1.5-1.7	Transparent	A sharp central ventral scale
IV	16-24	1.3-2.5	Opaque	No visible lateral root primordia
V	22	2.5	Opaque	Obvious lateral root primordia

8.5. EXPLANT AGE

Plant age and the degree of differentiation of tissues are often inter-related and produce interactive effects *in vitro*. Both the size and degree of development of certain organs, particularly cotyledons, hypocotyls and epicotyls (Fig. 10.10), depends on plant age. Thus the direct formation of adventitious shoots has been found to occur most readily from *Pinus glauca* cotyledons excised from 7-8 day-old seedlings. Prior to that the rate of shoot formation was slower, and after 8 days, the capacity of the explants to produce shoots declined rapidly (Toivonen and Kartha, 1988). A higher proportion of *Cucumis melo* tissue cultures produced adventitious shoots when they were initiated from small leaves (0.3-0.5 mm) from young (14 day-old) seedlings than from leaves of the same size from older seedlings (21, 28, and 35 days), or from larger leaves (0.6-0.9 mm or 1.0-1.2 mm) from seedlings of any of these ages (Kathal *et al.,* 1988).

8.6 PERIOD OF CULTURE

The period of time during which callus tissues have been maintained in culture commonly influences their morphogenetic potential. There is often a short period (one or more subcultures) during which regeneration increases (e.g. Reinert and Backs, 1968; Reinert *et al.,* 1971). This is followed by a period of high regenerative ability after which the capacity of the tissues to undergo regeneration often declines (see below). Adventitious shoot formation from *Arabidopsis* callus was increased if the normal subculture interval of 4 weeks was increased to 8 weeks just before transfer to a regeneration medium (Negrutiu and Jacobs, 1978).

8.6.1. Loss of regenerative ability

Some callus tissues retain a capacity to regenerate shoots in response to well-known stimuli, over long periods. Stimart *et al.* (1980) for instance, obtained shoot regeneration from *Lilium* callus maintained in culture for 3 years, and Sheridan (1974) found that a

regenerative capacity was still present in callus of this species after 8 years of subculturing. Callus of *Dioscorea* (Grewal *et al.,* 1977), of *Solanum nigrum* (Mandal and Gadgil, 1979), of *Datura innoxia* (Forche *et al.,* 1981) and of *Lavendula angustifolia* (Webb *et al.,* 1984) maintained a morphogenetic capacity for at least two years; corn and oat calluses have been reported that remained morphogenetically competent for up to 18 months (Rice *et al.,* 1979). The shoot-forming capacity of root and embryo callus of rice can be extended over a very long period if the callus is cultured with 2% sucrose plus either 3% mannitol or 3% sorbitol in the medium (Kavi Kishor and Reddy, 1986). However in conventional media, the facility with which regeneration may be induced from cultured plant cells very commonly decreases as the tissue is serially subcultured. The reasons for the loss of regenerative ability are still uncertain and may vary in different circumstances. Several theories have been advanced in the past to explain the effect:

- **Genetic variation in the cell population.** One widely-held theory is that cells are preferentially selected during culture for their ability to proliferate most rapidly under the chosen cultural conditions, and that this attribute is incompatible with the ability to undergo regeneration. Such a selection pressure will operate whenever there is appropriate genetic variation in the cell population, and can result in the loss of cells retaining the activated genetic information for totipotency (Sacristan and Melchers, 1969; Smith and Street, 1974; Orton, 1980).
- **The existence of a substance promoting regeneration,** which is present in freshly isolated explants and which slowly diminishes during growth *in vitro* (Reinert and Backs, 1968). The converse of this hypothesis would be that an inhibitor of regeneration accumulates with time.
- **An epigenetic change in the cultured cells.** The genotype of cells is essentially conserved, but mechanisms regulating the genetic information governing regeneration become inoperative or lost during subculture. The tissue could be considered to have become dedifferentiated with respect to regeneration (Rice *et al.,* 1979).

These hypotheses represent two contrasting ideas: either that non-competent cells have permanently lost their totipotency or that morphogenetic competence has only been impaired.

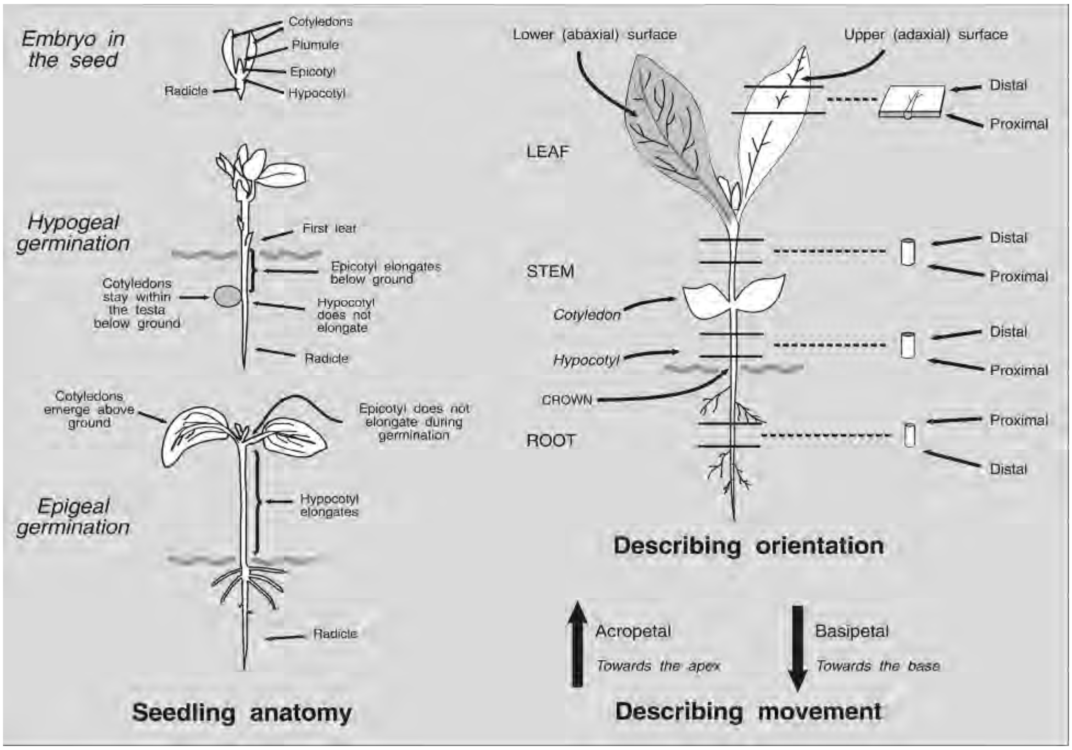

Fig. 10.10 Explant terminology – seedling explants and explant orientation.

Genetic variation as a cause.

There is considerable evidence to show that the *in vitro* culture of organised cells or tissues can lead to the formation of sufficient genetic variation in the resulting cell population for selection to be possible, leading to the accumulation of incompetent genotypes. Although the natural (or artificially-induced) occurrence of cells having aneuploid chromosome numbers does not necessarily prevent regeneration in calluses (Chapter 1), it seems clear that gross variations in the genetic make-up of callus cells will prevent plant regeneration (Rice and Carlson, 1975). In tobacco, Novak and Vyskot (1975) and Nuti Ronchi *et al.* (1981) found that many shoots or plants regenerated with aneuploid chromosome numbers, were weak and died in the course of development. The chromosome complements were presumably too imbalanced to permit survival. Moreover, aneuploidy is only one of the genetic changes that can be induced by callus and cell culture and so other karyotypic alterations may be even more responsible.

The argument that morphogenetic capacity is lost during an active selection for cells capable of high rates of growth, has usually been based on the observation that regenerative capacity of a cell population decreases as karyotypic abnormalities increase. Loss of regenerative ability is not always dependent on the occurrence of karyological diversity in a cell population. For example, one race of *Brachycome* lost its capacity to regenerate embryoids in 2 months, and its ability to produce roots, in 7 months. The cells remained strictly diploid over the whole of this period and beyond (Gould, 1978).

Epigenetic change as a cause.

Rather than being due to the disappearance by selection of cells possessing the necesssary genetic information for organogenesis, loss of morphogenetic potential may be caused by a changed pattern of gene expression brought about by the cultural environment, and may be an epigenetic phenomenon (as, some would believe, are habituation and physiological ageing).

In the experiments of Northcote (1979), bean tissue remained undifferentiated on a 'maintenance' medium containing 2,4-D, and normally differentiated on an 'induction' medium containing NAA (no 2,4-D) and the cytokinin kinetin. However, after more than four transfers on the maintenance medium, the ability of the tissue to differentiate on the induction medium progressively declined. Cloning experiments showed that selection for the inability to differentiate, was not being practised, and after about 30 transfers on maintenance medium, the ability of the cells to differentiate was often restored quite suddenly over one transfer. Other tests indicated no loss of genetic information from the cell population, which simply did not respond to the normal amounts of NAA and kinetin.

Recalcitrance or the non-regenerative condition of callus could be caused by the lack of direct effects of media components, particularly growth regulators, on genetic expression. One possibility is that genes responsible for regeneration become silenced by methylation *in vitro* and so incapable of expression. Differentiation and dedifferentiation have been shown to be related to the methylation and demethylation of the cytosine residues of DNA. The differentiation of carrot tissues has been shown to be characterised by a methylation pattern, and during the linear growth of carrot callus, methylation increases whilst during the stationary phase it decreases. The process has been shown to be influenced by growth regulators, with auxin leading to an increased methylation and cytokinin blocking changes to methylation. (de Klerk *et al.,* 1997). Such methylation is also considered to provide a marker of ageing, phase changes and reinvigoration of *Pinus radiata* (Faga, 2002 a,b,c). However, whilst, methylated DNA results in the gene being silenced, silencing is due also to other mechanisms (see below), the methylation process apparently acting to ensure that the gene remains silenced. Demethylation only occurs once the silenced gene has begun to be transcribed (Alberts *et al.,* 2002). The concept of silenced genes in recalcitrance may be more complex than a question of the methylation of DNA. For example, gene silencing also concerns the methylation of lysine 9 of the H3 histone tail of the nucleosome which results in gene silencing through the remodelling of the euchromatin into heterochromatin (Alberts *et al.,* 2002; Rice and Sedat, 2001). If we deal with the process of competence and recalcitrance to regenerate in relation to the cell cycle, it is possible to see that competent calluses can readily respond to plant growth regulator treatment, enter the cell cycle and move into the regeneration phase by the regulaton of genes – possibly by methylation and demethylation of the relevant ones. This would involve receiving a signal at a moment in G1 prior to START. However, some calluses need a

growth regulator shock to be induced to regenerate. In this case it is possible that the genes have been silenced in a way that involves DNA methylation of both the genes and the H3 histone residues of the nucleosomes so resulting in the relevant genes being held in a minimal form of heterochromatin, this state being reversed by the e.g. auxin shock.

The third situation relating to recalcitrance requires that the callus cells are locked in GO and cannot enter G1 to be able to cycle and to receive the growth regulator signal. This could happen if phosphorylated Rb protein is bound to and inhibits the eIF proteins which normally induce the binding of m-RNA to ribosomes. The Rb protein could then interact with HP1 (methyl lysine binding proteins) and CLF (*Arabidopsis* SET-domain CURLY LEAF protein) proteins. Rb can be found at the boundary between euchromatin and heterochromatin which results in the silencing of the euchromatic genes, In this situation, it is possible that such silenced genes would result in the cells being contained in G0 and could not be activated by plant growth regulator signals (Gahan, 2006).

A more recent development currently being investigated is RNA interference, which could have important roles to play in the process of recalcitrance Double-stranded interference RNA is involved with gene silencing through the binding of one strand of the dsRNA either directly to the DNA or to mRNA, which is subsequently destroyed (Novina and Sharp, 2004; Matzke and Matzke, 2000); it is involved also with the gene silencing and the formation of heterochromatin (Martinnsen *et al.,* 2004; Lipmann and Martinssen, 2004). In plants, such dsRNA is targeted to CpG islands within a promoter region of the chromosome and can also induce RNA–directed DNA methylation (Pelissier and Wasseneger, 2000; Hamilton *et al*, 2002; Zilberman *et al.,* 2003). DNA methylation has been demonstrated to occur both in DNA and the histone H3 lysine in human cells (Kawasaki and Taira, 2004). Such RNA has also been implicated in aspects of heterochromatin (Matzke and Matzke, 2000).

There have been some reports of the restoration of regeneration in 'old' cultures previously considered to be morphogenetically incompetent. A 17-year old cell line of tobacco, which had lost its ability to regenerate plants for 8 years, was re-induced to give rise to shoots (and hence plantlets) by a series of subcultures on a shoot-inducing medium (Rice *et al.,* 1979). At each subculture sectors of the callus which were greener and more compact were selected for transfer. During the third subculture some cultures formed abnormal leaf-like structures and after further rounds of selection, normal shoots were eventually obtained. Traynor and Flashman (1981) obtained shoot formation in tobacco callus lines, which had been maintained for 3-17 years on various media by subculture on a medium containing low levels of IAA (*e.g.*, 0.05 mg/l) and high (15-25 mg/l) concentrations of the cytokinin 2-iP.

Although media alterations did not restore the morphogenetic capacity of 'old' *Solanum laciniatum* callus, Chandler *et al.* (1982) noticed that whereas large explants from young cultures initiated shoots and small ones did not, the reverse was true of old cultures. Shoot formation was also initiated much more slowly in cultures of old tissue. The authors suggested that these observations were consistent with the gradual accumulation of an inhibitor of regeneration in the tissues. In other circumstances it is possible to conjecture on the absence of a promotor being responsible for the failure of regeneration. Sometimes shoot regeneration from primary callus only occurs when the original explant is still attached (e.g. from *Chrysanthemum* leaves - Khalid *et al.,* 1989). This implies a strong possibility of the need for either adjacent vascular tissue elements to be present in the explant or the presence of cells which already carry the activated programme for vascular tissue formation and can ready form vascular elements *in vitro* (Rana and Gahan, 1982). Thus, in the case of the data of Chandler *et al.* (1982), in younger explants, substances diffusing from vascular tissue, but which are not present in the cells of the older vascular tissue, could aid in the shoot initiation process.

8.6.2. Altered morphogenetic potential.

Plant cells may not only show a gradual loss of regenerative capacity the longer they are cultured, but also an altered morphogenetic potential. There are many reports in the literature of, for example, altered sensitivity to applied growth substances for shoot bud initiation, or tissues forming only roots where previously both shoot and roots had been differentiated. These events could also be explained in terms of a new epigenetic gene expression and/or an altered pattern of endogenous hormone synthesis, although an explanation based on genuine genetic change is usually preferred (see Volume 2).

9. THE NATURE OF AN EXPLANT

The manner in which explant derivation can influence growth and morphogenetic potential has been partly covered in the preceding paragraphs where it will be seen that variation in competence or regenerative capacity can be found between explants from different organs and also between tissues within the same organ. The highest rate of micropropagation will often depend not only on the selection of the most suitable explant, but also on the discovery of the correct combination of growth regulators, and/or the best nutritional composition of the medium (Mathews, 1987) for that organ or tissue. The results obtained from any one type of explant can vary between even closely related genotypes. In wheat, only embryo axis calli of the cultivar 'Buch Napost' produced adventitious shoots, but they could be obtained from embryo axis and plumule calli in the hybrid cultivar 'H81199' (Elena and Ginzo, 1988).

For meristem, shoot, or node culture, there is usually only a limited range of explants which can be used. Survival and growth of shoot tips from the apex of growing shoots are generally better than those of lateral buds, although the latter can frequently be employed satisfactorily. In some plants (e.g. *Eucalyptus*), a node bearing a bud is a more reliable initial explant for shoot cultures than a dissected shoot apex.

Explants from hairy organs are usually difficult to decontaminate. This was the case with hairy softwood explants of *Leucospermum* (Proteaceae), which also failed to grow, probably because the prominent indumentum caused explants to be perpetually covered by a layer of liquid (Ben-Jaacov and Jacobs, 1986).

9.1. TYPE AND POSITION

9.1.1. Explants for adventitious shoot regeneration

Choice of the correct explant is important when propagation is to be based on direct or indirect shoot initiation. Although in some species, explants from many organs are capable of producing adventitious shoots, it is usually found that those excised from different organs or from different tissues within an organ, vary strongly in morphogenetic capacity. Thus in *Brassica carinata*, 86% of cotyledon explants produced shoots, 74% of hypocotyl segments, but only 26% of root segments (Jaiswal *et al.,* 1987).

In such circumstances, it would be necessary, for efficient micropropagation, to select the type which had the highest capacity for regeneration. However, in some plants the availability of competent tissues can be limited, so that there is little choice. One of the best examples is that of direct or indirect embryogenesis, where ovules, nucellus tissue, nucellar embryos, zygotic embryos or parts of young seedlings, or very young leaves, commonly provide the best explants for obtaining somatic embryos or embryogenic callus.

In *Jasminium*, where explants show a remarkable capacity to produce callus *in vitro*, Khoder *et al.* (1979) obtained callus cultures from the shoot apex, petals, anthers, stem internodes, and flower peduncles, but shoot regeneration was only obtained from shoot apex callus. Callus derived from seedlings and inflorescence explants of various *Cymbopogon* species had a higher morphogenetic capacity than that arising from seeds, culms, roots or rhizomes (Jagadish Chandra and Sreenath, 1982). Different regenerative capacities are often found in mature leaf tissues. To obtain callus formation and/or organogenesis, it is frequently beneficial to include a portion of midrib or leaf vein in pieces of leaf lamina used as explants (Kato, 1974). In *Armoracia rusticana*, more direct shoot formation took place on leaf segments with part of a lateral vein, than on those which included part of the midrib (Grecka, 1987).

9.1.2. Accessory organs.

The presence of a leaf petiole on 1 cm node explants of *Rosa hybrida* inhibited shoot growth *in vitro* (Mederos and Rodriguez Enrquez, 1987), but the greatest potential for direct shoot regeneration from the basal portion of the ray florets of *Chrysanthemum morifolium* occurred when a pistil was still attached (Khalid *et al.,* 1989). Explants taken from the base of the immature floral stems within tulip bulbs generally produced more adventitious shoots than segments bearing nodes (Taeb and Alderson, 1987). In these experiments an interaction was found between explant position and the time of year at which explants were placed into culture. Explants from the base or the first node of the stem produced less shoots when cut from stored bulbs during November to March, whereas production of shoots from the second node was enhanced during this period.

9.1.3. Topophysis

Strictly positional effects on regeneration (e.g. where similar buds from the top and bottom of a plant behave differently *in vitro*) are examples of topophysis (an effect of position upon the observed characteristics of a plant). In experiments with trees, positional effects are sometimes further divided into cyclophysis (differential effects exhibited by parts of the same age, but different position) and periphysis (distinctive characteristics shown by parts of the same age and position which have been subjected to different physical exposure) (Oleson, 1978; Pierik, 1987).

9.1.4. Growth regulator requirements

Explants of different origin react to growth regulators in distinct ways and, as with choice of explant, this is particularly obvious in the formation of adventitious shoots. In some species, one type of explant may respond to a particular growth regulator treatment while another does not; in others the time taken to respond may vary. The proximal halves of the leaf lamina of *Citrus mitis* developed adventitious buds in 3 weeks when cultured with 2 mg/l BA, but the distal halves of the same leaves only regenerated shoots after at least 10 weeks in culture (Sim *et al.,* 1989).

The optimum dosage of growth regulators to induce regeneration often varies according to the size and type of explant from a single plant. In *Brassica carinata* (Jaiswal *et al.,* 1987), the greatest number of adventitious shoots was obtained by adding to MS medium 5.0 μM BA for cotyledon explants, 0.5 μM BA for hypocotyl explants, and 1.0 μM BA for root sections. The best medium for regenerating shoots from cotyledon-derived callus of *Lotononis bainesii*, contained 1 mg/l NAA and either 0.1 or 1 mg/l BA, while for leaf callus it was necessary to use 0.01 mg/l NAA and 0.1 mg/l BA (Bovo *et al.,* 1986).

In some instances it may even be advantageous to vary the regulatory compounds used for different kinds of explant. Adventitious shoots were obtained in greatest numbers from petiole sections of *Oxalis tuberosa* using 3 mg/l BA and 3 mg/l NAA, but the best regeneration from internode sections occurred when they were grown on a medium with 3 mg/l zeatin plus 3 mg/l NAA (Khan *et al.,* 1988).

9.1.5. Natural growth factors

It is very probable that variations in the levels of endogenous growth substances are often responsible for either the success or failure of tissue cultures. Many of the influences of mother plant and explant origin discussed on previous pages, could be interpreted in this way. The hypothesis seems particularly relevant to the varying morphogenetic potential of explants of different origins, or those from mother plants in different stages of growth, especially where a morphogenetic gradient can be demonstrated.

Seedling cotyledons can be a source of growth factors (Kamisaka and Shibata, 1977) and in many species, explants from the epicotyl or hypocotyl of seedlings have a high morphogenetic potential. Is this because these tissues are especially juvenile, or because the cotyledons are a source of a diffusible growth promoting substance? The possibility that the latter might be the true explanation is well demonstrated by the potential of epicotyl or root sections of *Citrus sinensis* ‘Valencia’ seedlings to produce buds. As is shown in Fig. 10.11, this decreased the further the explants were from the cotyledon (Burger and Hackett, 1986).

9.1.6. Size

There is frequently an optimum size for explants used to initiate tissue cultures. Very small explants, whether they are shoot (or meristem) tips, fragments of whole plant tissues, or pieces of callus, do not survive well in culture, but large explants may be difficult to decontaminate effectively or are less easily manipulated. A good example is provided by the work of Davies and Dale (1979) with leaf discs of *Solanum laciniatum*. Those smaller than 2 mm in diameter showed a high mortality rate, although if they survived they ultimately produced as many shoots as 5 mm disks. Disks 10 mm across showed the same basic type of growth pattern as 5 mm ones, but often developed convolutions and thereby lost some contact with the medium. Shoot tip explants of apple with 4-6 leaves were less liable to vitrification than smaller ones with only two apical leaves (Standardi and Micheli, 1988).

9.1.7. Direct and indirect regeneration

Direct and indirect regeneration on explants also frequently depends on explant size. In *Brassica oleracea* small disks of vars. *fruticosa* and *acephela* showed the greatest increase in area and fresh weight, but the lowest frequency of root production (Dunwell, 1981b); and 7 mm disks of var. *italica* leaves produced 10 times more shoots per disk than 4 mm ones (Lazzeri and Dunwell, 1986). Similarly, as stem internode sections of *Brassica monnieri* were increased in length from 1 to 4 mm, a greater proportion of them formed shoot buds, and the

number of shoots per explant increased. The efficiency of shoot production (i.e. the number of shoots formed per mm of internode) was however inversely proportional to explant length, except that those smaller than 1 mm failed to show any organ formation (Thakur and Ganapathy, 1978). The same situation was found in *Hyacinthus*, where the number of bulblets per bulb scale explant increased as explant length was enlarged from 1 cm to 3 cm, although the number of bulblets per unit length of explant was thereby decreased (Paek, 1982). Hypocotyl explants of *Albizia richardiana* which were 1 mm in length only produced a large amount of callus, whereas shoot buds were produced on 10 mm sections (Tomar and Gupta, 1988).

On cultured pieces of *Convolvulus* roots, where new shoots arose from pre-existing meristematic primordia, the number of regenerated shoots and roots per explant increased as length increased, but here the number of roots and shoots produced per unit length of explant was largely independent of explant size, except that very small segments (1.5 mm) were again incapable of organ formation (Bonnett and Torrey, 1965).

Root formation from *Populus callus* was found to depend on the size of the callus pieces subcultured (Winton, 1968). The greatest number of roots occurred on the largest callus pieces, grown from the biggest explants, of the oldest tissue sources. This indicates that root initiation was influenced by the degree of tissue differentiation when it is likely that xylem elements were already present in the callus and set a pattern for root differentiation.

By contrast the frequency of leaf (shoot) formation in *Arabidopsis* callus was highest when small callus pieces (75 mg) were used as inocula on an induction medium. A larger inoculum size (250 mg) favoured continued callus proliferation at the expense of regeneration (Negrutiu and Jacobs, 1978).

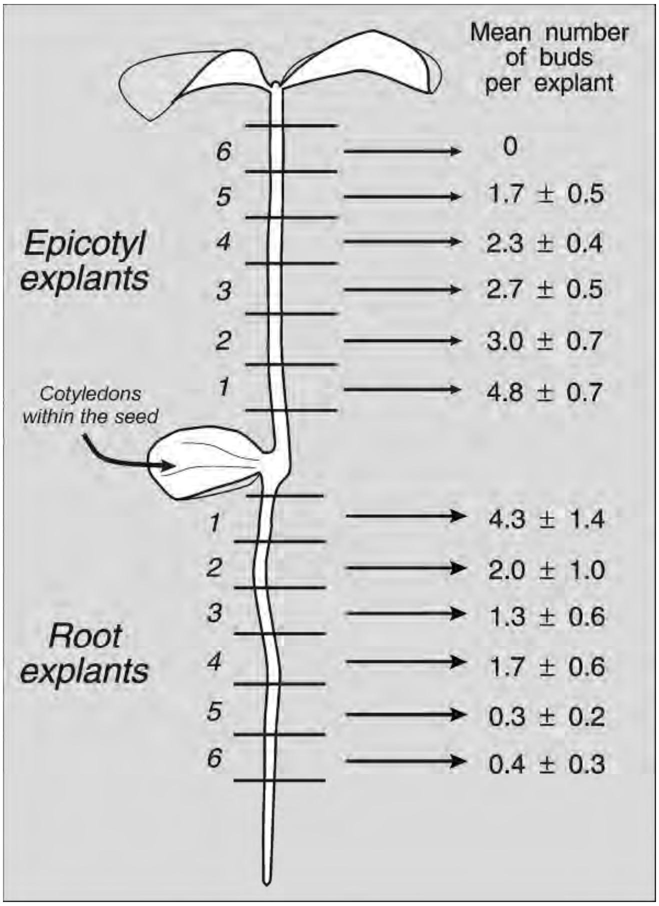

Fig. 10.11 The number of shoot buds formed on successive explants (1 cm-long) from the epicotyls and roots of *Citrus* seedlings [after Burger and Hackett, 1986].

10. METHOD OF CULTURE

10.1. INOCULATION DENSITY

The need for a minimum inoculation density to induce cell or tissue growth *in vitro* has been mentioned before. Besides affecting cell growth, inoculation density is occasionally reported to affect cell differentiation, regeneration or shoot proliferation.

10.2. EVIDENCE FOR CONDITIONING FACTORS

After incubating suspension cultures of wild carrot for 10 days, both the number of somatic embryos formed and the growth stage, which the embryos had reached, were found to depend on the amount of inoculum used to start the cultures. Increasing dilutions of the inoculum resulted in undifferentiated cell growth. Not only were less embryos formed than would have been predicted from the reduced inoculum size, but those present were progressively smaller in size and less well-developed (Halperin, 1967). Inoculum size was also found (by Verma and Durzan, 1980) to be an important factor in carrot embryogenesis, and Hari (1980) showed that cells do not reach their full embryonic potential at either low or high densities. It was concluded that cells produced an active metabolite (present in conditioned media) essential for complete embryogenesis. This is further confirmed by experiments showing that too low a carrot cell concentration (less than 10^4 cells) resulted in cell death by a programmed cell death pathway, though the addition of a conditioned medium allowed the cells to remain alive (McCabe *et al.,* 1997). The nature of the factors involved is not clear. However, the fact that cells release newly synthesised DNA into the medium may be significant - such DNA appears to freely enter cells where it can be expressed (Gahan, 2004). It has been proposed that this DNA can signal between cells (Adams *et al.,* 1997) and so may be an important factor in the culture process, being in too small an amount to have an impact when the cell number is too low.

By culturing pieces of embryogenic *Oryza sativa* callus of different weights in a constant volume of medium, or pieces of constant weight in different volumes of medium, Raghava Ram and Nabors (1985) found that the optimum rate of plant regeneration occurred when the ratio of callus to medium was 6.5-7.5 mg ml^{-1}. Plant regeneration was considerably reduced and callus growth increased when larger amounts of callus were used: the tissue often died when smaller pieces of callus were explanted. These results were thought to be caused by the release of a conditioning factor into the medium.

An explant density effect was reported by Pierik and Steegmans (1972). When ten seedlings of a *Cattleya orchid* were inoculated into a single flask of liquid medium, they formed protocorms and plantlets, but no protocorms were produced when the inoculum was only two plants per flask. Similarly, lateral buds of asparagus on MS medium produced more shoots and roots when many were put into the same flask. The effect was thought to be due to a substance diffusing from the explants, because growth could be promoted in the same fashion at lower densities by addition of an auxin and cytokinin (Matsubara, 1973; Matsubara and Clore, 1974).

In culturing tuber disks of *Helianthus tuberosus* to study the formation of tracheids, Phillips and Dodds (1977) found by accident that the number of elements produced was greatly increased when the volume of medium per disk placed on filter paper was reduced to below 0.2 ml. Above this figure (e.g. 0.5 ml per disk) there was a sharp decrease. Similar results were found with explants of *Pisum sativum* (Rana and Gahan, 1982) when the cells converted to tracheids were greater at the base of the explants decreasing towards the top. The effect was not due to medium conditioning but appears to involve the need for a polarity of movement of components from the medium rather than a total immersion of the explant.

10.3. NUTRIENT SUPPLY

The smaller the size of an explant, or the number of explants on a given quantity of medium, the greater should be the availability of nutrients to the cultured tissues; but a high volume of medium per explant may not necessarily result in the best rate of propagation, for the condition is akin to inoculating suspension or callus cultures at low densities, and essential conditioning factors may become limiting. That conditioning factors can be important with whole plant explants is suggested by results of Dunwell (1981a): an interaction between medium composition and explant density was noted with *Brassica oleracea* leaf disk explants. When 5 disks were placed on a dish, glutamine increased growth on MS medium, whereas when there were 10 disks per dish, it reduced growth.

One result indicating that nutrient supply can be important is that of Chun *et al.* (1986). The accumulation of dry weight and the number of axillary shoots per explant in Stage II *Populus* shoot cultures was best when only one shoot was subcultured into each vessel, but the greatest number of shoots per vessel (i.e. the maximum rate of practical shoot multiplication), occurred when 2 or 3 shoots were explanted into each container. The effect was independent of the size of the container used, and as the amount of medium was constant throughout, Chun *et al.,* concluded that competition for nutrients was able to limit growth and shoot proliferation.

10.4. EFFECTS OF POLARITY

10.4.1. The orientation of explants

Positions on entire or sectioned organs of plants are given relative to the crown (the junction of shoot and root). The morphologically uppermost surface of shoot or leaf explants is said to be distal; that of root explants proximal to this point. The lower side of leaves is said to be abaxial and the upper side adaxial (Fig. 10.10).

Polarity depends on cells having a capacity for the electrogenic exchange of hydrogen ions (H^+) and potassium ions at the plasma membrane (plasmalemma). Auxin-promoted H^+ efflux in basal cells probably sets up and maintains an electrical potential difference in tissues (positive at the apex, negative at the base), which maintains and reinforces auxin transport (Raven, 1979). Excision depolarises shoot and root tissue, but subsequent washing, ageing or incubation in an aqueous aerated medium restores the cell potential and allows re-polarization to take place, normally within 4-6 h in roots and within 12-16 h in pea epicotyl tissue (Hanson and Trewavas, 1982). Cultured explants usually show a marked polarity in cell proliferation and regeneration. This may be related to both the position and/or orientation which the organ or piece of tissue had on the intact plant, and also to its orientation within the culture vessel. Polarity of regeneration varies in different genotypes (even between varieties within a species) and can sometimes be reversed by growth regulator treatments.

10.4.2. Position on the mother plant

Polarity in stem and root sections. Stem and root pieces normally produce shoots from the morphologically uppermost cut end, and roots from the lower end. Segments of dandelion root, for example, show a very strong polarity (Bowes, 1976a). This is first apparent as a polarity in callus formation. At the distal end of a root segment the phloem proliferates markedly, producing a pronounced callus from which adventitious roots arise. There is much less callus formation at the proximal end of the explant where shoots arise adventitiously from superficial callus-derived meristems. Polarity of regeneration developed slowly in cultured *Epilobium* root segments: buds and roots were formed at first along the whole length of cuttings, but gradually bud initiation came to be localised towards the proximal ends, and root intiation towards the distal ends (Emery, 1955). When the hypocotyls of *Capsicum* seedlings were cut into 6 equal segments, shoots were only produced from apical sections, central sections produced mostly roots, while abundant callus growth occurred on basal segments (Fari and Czako, 1981). Segments of the inflorescence stalk of *Gladiolus* produce callus and roots at the basal end, followed by the formation of buds and cormlets at the distal end (Ziv *et al.,* 1970).

Gradients in regeneration. It is usually found that when a complete organ is cut into pieces, the various segments differ in their morphogenetic capability. There is frequently a gradient in regeneration in progressive sections. For example, in *Helianthus annuus*, 5 mm sections from the middle of the hypocotyl have been found to be the best explants for obtaining callus capable of regenerating adventitious shoots (Greco *et al.,* 1984). The morphogenetic capacity of sections of *Citrus sinensis*, epicotyls and roots has been mentioned previously (Fig. 10.11). The observed gradients were apparent on Murashige and Tucker (1969) medium containg 2 mg/l BA and 0.02 mg/l NAA; less shoots were formed on all sections, and the gradients were not apparent when the concentration of both regulants was 2 mg/l (Burger and Hackett, 1986).

Pedicel sections of the flowers of *Lilium longiflorum* were found by Liu and Burger (1986) to show a gradient in the formation of buds. The section taken from nearest the receptacle (the most distal section) formed the greatest number (nearly 5 times more than the next adjacent section), especially if it was placed upside down on the medium (see below).

Polarity in whole leaves and leaf pieces. Although in some species adventitious buds can be formed over the entire surface of detached leaves, shoots and roots more usually arise at the base of leaves or leaf fragments, i.e. at the lower proximal end (i.e. that nearer the stem - see Fig. 10.10). This

polarity in the capacity of leaf tissues to regenerate adventitious shoots, can be seen in the relative number of shoot buds produced over the surface of an entire leaf, but is more commonly manifested when leaves are cut into sections. The regenerative capacity of leaf fragments cut from detached leaves is then usually greater in those from the proximal end than in those from the distal region. This may occur in some plants because the proximal region of the leaf contains more meristematic cells than distal regions. There was a higher rate of shoot regeneration in sections cut from the proximal half of *Malus* leaves (Welander, 1988) while in *Echeveria elegans* (Crassulaceae), shoots were formed only in the proximal region of entire detached leaves and at the proximal ends of half-leaf segments (Raju and Mann, 1971). In this plant, shoot formation from the base of distal leaf halves was much slower than that from proximal halves. Similarly, the proximal halves of the leaves of *Citrus mitis* seedlings formed direct adventitious shoots after 3½ weeks of culture on a medium containing 2 mg/l BA, but shoot regeneration from the distal halves was much slower and only started after 10 weeks of culture: only a few shoots had formed after 12 weeks (Sim *et al.,* 1989).

Callus originating from cotyledon fragments proximal to embryo axes of *Olea europea* was most likely to regenerate shoots (Canas and Benbadis, 1988) and regeneration occurred on more of the explants (81%) cut from the basal region of Douglas fir cotyledons than on explants taken from the middle (69%) or distal parts (52%) and grown under equivalent conditions (Cheng, 1976, 1977). However in *Heloniopsis orientalis* (Liliaceae), shoot buds are formed more frequently from the distal parts (see Fig. 10.10) of mature leaves than from proximal segments (Kato, 1974).

A greater number of protocorm-like bodies were produced from explants of proximal leaf tissue in *Phalaenopsis* orchids, than from distal explants (Tanaka *et al.,* 1975). Sections cut from the inner part of head lettuce leaves tended to produce callus and only a few shoots, while those from the periphery of leaves all gave rise to shoots (Koevary *et al.,* 1978).

Differences between leaf surfaces. Some plants are able to produce callus or to regenerate organs from either leaf surface, but from the Mexican Snowball plant, Raju and Mann (1970) obtained shoots only on the adaxial surface of explants. In *Phalaenopsis* and *Vanda* orchids, protocorms were formed from primordia developed on the adaxial surfaces of either small leaf fragments or whole immature leaves (Tanaka *et al.,* 1975). Contrary to this, regenerative callus only arose from the lower (abaxial) epidermis of *Bryophyllum daigremontianum*, and other leaf tissues never produced buds under the same culture conditions (Bigot, 1976). The abaxial surface of *Ipomoea batatas* leaves formed more embryogenic callus than the adaxial surface (Liu and Cantliffe, 1984). The leaf surface from which adventitious shoots may arise is usually genetically determined. Yanagawa and Sakanishi (1980) showed that regenerative capacity was high on the adaxial surface of bulb scale explants from plants in the Liliaceae, and on the abaxial surface of Amaryllidaceae. Shoot regeneration therefore occurs most frequently on the side of the scale in which the vascular bundles are sited, indicating that the bundles may provide cell division stimuli.

Explants taken from the bulb scales of bulbous plants regenerate adventitious shoots and bulblets more freely if cut from the basal (proximal) part of the bulb scale rather than from the distal region (Hackett, 1969). Adventitious shoot formation is always much higher if some part of the basal plate of the bulb is included with the scale explant. *Lilium rubellum* can be propagated from bulblets formed directly on leaf segments. Explants derived from the base of the leaf had a higher regenerative capacity than those taken from the leaf apex. This may be due, in part, to the fact that the cells at the base of the leaf are the youngest. In this plant, leaves from the upper part of the stem produced bulblets more readily than those from the base. The capacity of leaves to form bulblets decreased with the approach of flowering and at, or shortly after flowering, it disappeared completely (Niimi and Onozawa, 1979).

10.4.3. Growth regulators and polarity

Natural polarity of regenerative events is normally supposed to be due to the movement of growth substances within plant tissues, particularly the polar (i.e. from the shoot apex towards the root tip) transport of IAA auxin (see Chapters 5 and 8). Thus IAA movement is naturally basipetal in the stem and acropetal in roots. These patterns may not be apparent in small tissue sections, which would explain why polarity of organogenesis is not usually found in very small explants, but tends to increase as explant size is increased (Thakur and Ganapathi, 1978). The addition of auxin and cytokinin to culture media may reinforce the normally observed polarity

of explants, induce regeneration from unresponsive parts of organs, or lead to the disappearance or reversal of polar trends through auxin accumulation at the basal end.

A reversal of polarity was instanced by Cutter (1962). *Zamioculcas zamiifolia* leaves or leaf pieces produced tuberous swellings from their proximal ends from which roots and one or more shoot buds arose. Regeneration could also be induced from a distal cut surface if it was spot-treated with a concentrated NAA solution (186 mg/l). Loss of polarity following the application of growth regulators has been instanced in cauliflower, rape and *Begonia*. On media without growth regulators, cauliflower petiole explants produced roots and shoots at their proximal pole, but this polarity was cancelled if 1.9 mg/l NAA was added to the medium, when numerous roots were formed all over the explant. Shoots formed all over the explant when 2.3 mg/l BA was administered instead (Margara, 1969).

Shoot formation occurred at the morphological base of rape flower stalks placed on media containing relatively low concentrations of auxin and cytokinin, but this polarity was not apparent when the concentration of growth regulants was increased (Margara and Leydecker, 1978). Cultured without growth regulators, disks cut from the leaves of *Begonia rex* regenerated a root at the base of the longest vein, but the presence of 5 mg/l kinetin abolished the polarity of organogenesis. Adventitious shoots then arose over the whole area of both sides of the leaf (Schraudolf and Reinert, 1959). Auxin and cytokinin became essential additions to Linsmaier and Skoog (1965) medium to produce bulblet, callus and root formation from explants cut from the distal (and normally non-regenerative) part of *Lilium longiflorum* bulb scales (Dinnis and Ascher, 1976). Legrand (1972) noticed that the polarity of shoot regeneration from *Endiva* leaf fragments was progressively reversed, the longer they were cultured in darkness.

Interactions between growth substances and the normal polarity of explants can be complex. For instance, in the work of Bigot *et al.* (1977), sections taken from tomato hypocotyls tended to have a greater capacity to produce shoots the nearer the root they were taken. The region of the hypocotyl to show maximum regenerative ability, varied according to the variety of plant and according to the growth regulators in the medium. There was a greater difference in the regenerative ability of distal and proximal ends when 2.1 mg/l IAA and 1.0 mg/l 2-iP were used together, than when 1 mg/l 2-iP was used alone. Zimmerman *et al.* (1988) found that more hyperhydric plants of *Petunia* were obtained from distal nodal segments of normal plants than from proximal segments.

A morphactin [n-butyl-9-hydroxyfluorene-9-carboxylic acid, Chapter 5 (16)] added to the medium at a concentration of 3.5 mM, caused the roots of *Heloniopsis* plantlets to swell and produce semi-organised buds at their extremities (Kato, 1976). When excised leaves were exposed to the compound, shoot buds were initiated over the entire surface, rather than in a polarised fashion (Kato, 1978). These results were thought to be consistent with the known ability of morphactins to bring about a redistribution of natural growth substances. Nevertheless, the major impact on polarity appears to be the manner of the accumulation of auxin. This will be further influenced by the source of the material which is being induced to either shoot or root (Marks, 1996; Marks and Simpson, 2000).

10.5. THE POSITIONING OF EXPLANTS *IN VITRO*

Growth or regeneration from explants can be influenced by the way that the explant is placed into or upon the medium. The effects noted may sometimes be due entirely to polarity; on other occasions they can be attributable to the effect of positioning on the availability of nutrients and growth regulators to those parts of the explant which are competent.

10.5.1. Embryo explants.

The supply of nutritional factors is often of critical importance when excised embryos are cultured. For instance, rye embryos all germinated precociously when placed by Dunwell and Cornish (1980) with their scutellum on the medium, but only 35% did so if the embryonic axis was in contact with it. Embryo explants of *Nuytsia* (a parasitic plant) developed as normal seedlings on a solidified White's medium if placed vertically with the hypocotyl partially embedded, but the cotyledons callused if they touched the agar (Nag and Johri, 1976). Embryos of pine and Douglas fir initiated adventitious buds much more freely and rapidly if cultured in an inverted position, with their cotyledonary ends in the medium, rather than their radical ends (Kadkade and Jopson, 1977). A greater surface of the explant is then in contact with the medium (Aitken-Christie *et al.,* 1985).

10.5 2. Leaf explants

Positioning can be particularly important with leaf explants. Leaf segments of *Gastaria* and *Haworthia* produced more somatic embryos when prone on agar than when placed upright (Beyl and Sharma, 1983). Discs cut from young leaves of the strawberry cultivar 'Redcoat', produced more adventitious shoots when their adaxial surface was kept in contact with the medium than when placed the other way up (Nehra *et al.,* 1988). Similar results were obtained with segments of *Malus domestica* and *Malus pumila* leaves. Welander (1988) suggested that one explanation, besides polarity, for this observation might be that the adaxial surface is the most responsive to nutrients being the last tissue of the leaf to cease growing and dividing. The use of cotyledons and first leaves of *Solanum aviculare* (Gahan *et al.,* 1994) also indicated a preference for the contact of the adaxial surface with the medium for the induction of xylogenesis, rooting and shooting, as well as somatic embryogenesis (Alizadeh and Mantell, 1991). Moreover it was possible to demonstrate the pathway of the movement of the growth regulators through the surface to the centre of the explant (Gahan *et al.,* 1994). However, the best multiple shoot formation in response to BA, adventitious root formation in response to the auxins NAA, IAA and IBA, and callus formation in response to 2,4-D and NAA, occurred when the abaxial surface of leaf sections of a *Populus* hybrid was in contact with the medium (Park and Son, 1988). Similarly somatic embryos are formed directly from cells of the abaxial epidermis of the cotyledons dissected from immature soybean seeds. Hepher *et al.* (1988) suggested that the embryogenic potential of this tissue was associated with its role as a nutrient transfer tissue.

10.5.3. Bulb scale explants.

Bulb scale explants of *Lilium longiflorum* produced far more bulblets per section when the abaxial (convex) surface was touching the medium than when their adaxial surface was in contact (Hackett, 1969). This result has been confirmed by Leshem *et al.* (1982) who obtained many more and larger bulbs, more roots and less callus, when the abaxial (rather than the adaxial) surface of scale segment was downwards on the medium. In all cases, bulblets developed only on the adaxial side of the scale. A similar result was described by Niederwieser and Vcelar (1990) with leaf sections of *Lachenalia* (Liliaceae) hybrids. Although adventitious buds were produced on the adaxial surface, regardless of the orientation of the explants, the best results occurred when the abaxial surface was in contact with the medium, because if the explants were the other way up, buds grew to form a hard callus-like tissue or were thickened and abnormal.

10.5.4. Stem and root explants

The organogenesis obtained on root or stem sections is also liable to differ according to whether the explants are normally orientated or inverted on the medium. Shoots regenerated from the upper (proximal) surface when dandelion root sections were placed distal side (i.e. the side that would normally be nearest to the root tip) downwards on the medium. Reversed, with the proximal side in contact with the agar, they produced a mound of callus from the upper (distal) surface (Booth and Satchuthananthavale, 1974). Adventitious roots formed from *Acinidia deliciosa* callus formed shoots at their proximal end if they were detached from the callus and inserted vertically with the root tip in the agar medium, but produced shoots along much of their length from the proximal end if placed horizontally (Revilla and Power, 1988).

When the basal ends of white spruce hypocotyl sections were placed in the medium, almost all the explants produced scale-like organs which could be subcultured to give buds and shoots, but only roots were formed on 50% of the sections if they were cultured with their apical ends downwards (Campbell and Durzan, 1978).

Discs cut from the inflorescence stalk of gladiolus developed callus and root primordia from their basal ends when that end was placed on the medium, but if explants were cultured in an inverted position, regeneration was more rapid. Roots were again formed at the proximal end (now uppermost), and one or two buds developed at the distal end (in the medium). Subsequent leaf growth turned the explant over. Subcultured correctly orientated on the same medium, explants eventually developed clusters of cormlets and one or two shoots (Ziv *et al.,* 1970). It was similarly advantageous to invert scape sections of asparagus (Takatori *et al.,* 1968) and *Narcissus* (Seabrook *et al.,* 1976) to obtain any morphogenetic response.

It has been suggested that inversion may overcome the polar transport of natural auxin, but this was not the case with tulip. Stalk explants elongated only when they were inverted on a medium containing IAA, and growth was due to basipetal (polar) auxin movement (Gabryszewska and Saniewski, 1983). Pierik and Steegmans (1975)

thought that the strong promotion of rooting from the proximal end of *Rhododendron* stem segments, when they were inverted on a solidified medium, was probably due to a better oxygen supply.

Explant position is not invariably important. Stem sections of olive produced callus and roots from the end immersed in the medium, regardless of whether it was proximal or distal (Scaramuzzi and de Gaetano, 1974); in *Echeveria elegans*, leaf freagments produced shoots always at the proximal cut end even if they were inverted (Raju and Mann, 1971); and direct shoot regeneration occurred to the same extent on petiole segments of *Saintpaulia*, regardless of their orientation (Harney and Knap, 1979).

10.5.5. Callus explants

Polarity in a callus explant is assumed to be from the medium vertically to the apex of the explant. However, if the callus contains vascular elements e.g. tracheids, procambial cells, these cells will influence the directions in which polarity will occur. It is possible that there are a number of mini-polarities formed as groups of cells become competent for organogenesis so imposing particular polarities on specific parts of the callus.

11. COMPETITION BETWEEN MERISTEMS

The first organogenetic meristems formed *in vitro* can inhibit the development of further structures of the same kind. New shoot meristems appear equidistantly, on the surface of the explants composed of thin cell layers (Tran Thanh Van *et al.,* 1974), suggesting that immediately a meristem is formed, it sends out a message inhibiting the initiation of another meristem in its near vicinity. That the inhibitor could be auxin, is suggested by experiments of Cassells (1979). The inhibiting effect of meristems on further regeneration and on the development of pre-formed organs, increases as meristems enlarge. Removing shootlets as they are formed may therefore facilitate continuous adventitious shoot production from explants or callus tissue, whereas if the tissue is left undisturbed, the total number of shoots may be small. Von Arnold and Eriksson (1978) found that out of 10-30 buds formed on Norway spruce embryo explants, only about 3 would elongate unless those which had reached 3-5 mm high were excised. The large number of shoots formed on *Pinus radiata* cultures were mutually inhibitory to further growth unless the nodular meristematic tissue from which the shoots had been produced was subdivided and the pieces moved to fresh medium (Aitkin *et al.,* 1981).

Competition can also occur between somatic embryos. Although many embryos were formed from scutellar callus of *Pennisetum americanum*, only 2-3 plantlets were produced from each explant if it was transferred to a 2,4-D-free medium in one piece. If the callus was divided into 4-5 pieces, one plant was generally produced from each segment (Vasil and Vasil, 1981). Gharyal and Maheshwari (1981) showed that direct embryoid formation would persist on *Albizia lebbek* hypocotyl explants, providing that recognisable embryos were continually removed, as they appeared, and were then cultured separately..

12. THE CONTROL OF DEVELOPMENT

The methods whereby epigenetic messages are unfolded are not fully understood (Wolffe and Matzke, 1999). Although large amounts of DNA may be present in the plant genome, much of it is not required for the organisation of the organism. This is very clear from the human genome in which only some 35,000 genes are involved in the organistion and functioning of man (Wolfsberg *et al.,* 2001; Malakoff, 2001). Thus, of the large variations of DNA content seen in plants (Bennett and Smith, 1976) very little is actually needed for the structuring and functioning of the plant. In *Arabidopsis thaliana* there are only a total of $10x10^7$ base pairs, which compares with $2x10^7$ in yeast. Normally, the DNA which is not involved in active gene transcription is present at interphase as clumps of heterochromatin whilst the transcribed DNA is present as euchromatin. The regulation of the DNA into readily transcribed and non-transcribed regions is affected by acetylation of the histones structuring the chromatin. Equally, the way in which the histones are acetylated will aid in the replication of the DNA (Voet *et al.,* 1999). The availability of genes for transcription will also depend upon the methylation of the cytosine residues which will result in the non-transcription of the DNA (Swartz, 1991; Voet *et al.,* 1999). Gene transcription arises when a specific general transcription factor or binding protein attaches to the

relevant gene site together with a pre-initiation protein complex at the TATA box site which will recruit the RNA polymerase necessary for the transcription to occur. However, this is further complicated by the association of either an enhancer protein or a silencer protein at a distance from the gene. The enhancer will activate the process whilst a silencer will suppress the gene activity.

Studies on the flowering process have given a lead as to what sort of genes need be researched when looking for those related to the control of root and shoot formation. Three classes of highly conserved, homeotic genes have been identified as controlling the production of the floral whorl in *Arabidopsis thaliana* and *Antirrhium*. (Coen, 1991; Honma and Goto, 2001). These highly conserved gene classes are not comparable to the homeoboxes of e.g. *Drosophila*, but to another family of regulatory genes called the MADS family which are found in both yeasts and vertebrates and which control the development of blocks of tissue. Such gene families are likely to be activated in plants by either light (Briggs and Liscum, 1997; Bowler, 1997:Smith, 2000) or growth regulators (Rogler and Dahmus, 1974; Domoney and Timmins, 1980; Davies and Hartmann, 1988; Estruch *et al.,* 1993; Hall and Smith, 1997; Thomas *et al.,* 1997). Most of the components of the main cell signalling systems found in animal cells have been found in plants i.e. heterotrimeric G-proteins (Hooley 1998; Jones, 1998; Plakidou-Dymock *et al.,* 1998), monomeric G-proteins (Moshkov *et al.,* 2003), cyclic nucleotides (Yamazaki, 1997; Trewavas, 1997), MAPkinases (Novikova *et al.,* 2000) and phospholipases (Roberts, 1997; Macdonald, 1997; Hooley, 1998) (see also Chapter 7). Recently, it has been demonstrated in *A. thaliana* that stem cell fate in the shoot apical meristem is controlled by a regulatory network including the CLAVATA (CLV) ligand system and the homeodomain protein WUSCHEL (WUS) (Brand *et al.,* 2000). A mechanistic link between the action of cytokinin and the CLV/WUS network (Liebfried *et al.,* 2005) has been demonstrated when it would appear that *ARABIDOPSIS RESPONSE REGULATOR* genes might negatively influence meristem size. Their repression by WUS could be important for normal meristem function.

REFERENCES

ABE T. & SASAHARA T. 1982 Genetical control of callus formation in rice. pp. 419-420 in Fujiwara (ed.) 1982 (q.v.).

ABO EL-NIL M.M. & WOCHOK Z. 1977 *In vitro* developmental responses of wild and full sib families of Douglas fir. Plant Physiol. 59, Suppl., 2.

ADAMS D.H., DIAZ N. & GAHAN P.B. 1997 *In vitro* stimulation by tumour cell media of [3H]thymidine incorporation by mouse spleen lymphocytes. Cell Biochem. Funct. 15, 119-126

AITKEN J., HORGAN K.J. & THORPE T.A. 1981 Influence of explant selection on the shoot-forming capacity of juvenile tissues of *Pinus radiata*. Can. J. Forest Res. 11, 112-117.

AITKEN-CHRISTIE J., JONES C. & BOND S. 1985 Wet and waxy shoots in radiata pine micropropagation. Acta Hortic. 166, 93-100.

ALBERTS B., BRAY A., JOHNSON J., LEWIS J., RAFF M., ROBERTS K. & WALTER P. 2002 *Molecular Biology of the Cell.* Garland Press, 4th Edition

ALIZADEH, S. & MANTELL, S.H. 1991 Early cellular events during direct somatic embryogenesis in cotyldon explants of *Solanum aviculare* Forst. Ann. Bot. 67, 257-263.

ARCIONI S. & MARIOTTI D. 1983 Tissue culture and plant regeneration in *Onobrychis viciaefolia* Scop. Z. Pflanzenzucht. 90, 192-197.

ARISUMI T. & FRAZIER I.C. 1968 Cytological and morphological evidence for the single-cell origin of vegetatively propagated shoots in thirteen species of *Saintpaulia* treated with colchicine. Proc. Am Soc. Hort. Sci., 93, 679-685.

ARMSTRONG C.L. & PHILLIPS R.L. 1988 Genetic and cytogenetic variation in plants regenerated from organogenic and friable, embryogenic tissue cultures of maize. Crop Sci. 28, 363-369.

ATTFIELD E.M. & EVANS P.K. 1991 Stages in the initiation of root and shoot organogenesis in cultured leaf explants of *Nicotiana tabacum* cv. Xanthi nc. J. Exp. Bot. 42, 59-63.

AUDERSET G., GAVILLET S., MICHELI J., O'ROURKE J., RIBAUX M. & MONCOUSIN Ch. 1994 Histological analysis and the evolution of biochemical markers during the *in vitro* rooting of *Malus domestica* Borkh.'Jork 9'. Adv. Hort. Sci. 8, 5-10.

BACKS-HÜSEMANN D. & REINERT J. 1970 Embryobildung durch isolierte Einzelzellen aus Gewebkulturen von *Daucus carota.* Protoplasma 70, 49-60

BARG R. & UMIEL N. 1977 Effects of sugar concentration of growth, greenery and shoot formation in callus cultures of four genetic lines of tobacco. Z. Pflanzenphysiol. 81, 161-166.

BARONCELLI S., BUIATTI M. & BENNICI A. 1973 Genetics of growth and differentiation *in vitro* of *B. oleracea* var. *botrytis*. I. Differences between 6 inbred lines. Z. Pflanzenzucht. 70, 99-107.

BARONCELLI S., BUIATTI M., BENNICI A. & PAGLIAI M. 1974 Genetics of growth and differentiation *in vitro* of *Brassica oleracea* var. *botrytis*. Z. Pflanzenzucht. 72, 275-282.

BARONCELLI S., BUIATTI M., BENNICI A., FOROUGHI-WEHR G., MIX B., GAUL H., TAGLIASACCHI A.M., LOIERO M. & GIORGI B. 1978 Genetic control of *in vitro* and *in vivo* growth in hexaploid wheat. I. Behaviour of ditelocentric lines. Z. Pflanzenzucht. 80, 109-116.

BAYLISS M.W. & DUNN S.D.M. 1979 Factors affecting callus formation from embryos of barley (*Hordeum vulgare*). Plant Sci. Lett. 14, 311-316.

BEN-JAACOV J. & JACOBS G. 1986 Establishing *Protea*, *Leucospermum* and *Serruria in vitro*. Acta Hortic. 185, 39-52.

BENNETT M. D. & SMITH J. B. 1976 Nuclear DNNA amounts in angiosperms. Phil. Trans. Roy. Soc. B. 274, 227-274.

BENTZ S.E., PARLIMAN B.J., TALBOTT H.-J. & ACKERMAN W.L. 1988 Factors affecting *in vitro* propagation of *Yucca glauca*. Plant Cell Tissue Organ Cult. 14, 111-120.

BEYL C.A. & SHARMA G.C. 1983 Picloram induced somatic embryogenesis in *Gasteria* and *Haworthia*. Plant Cell Tissue Organ Cult. 2, 123-132.

BIGOT C. 1976 Bourgeonnement *in vitro* à partir d'épiderme séparé de feuille de *Bryophyllum daigremontianum* (Crassulacées). Can. J. Bot. 54, 852-867.

BIGOT C., OHKI S. & MOUSSEAU J. 1977 Experimental data for a strategy for the improvement of the shoot forming capacity *in vitro*. Acta Hortic. 78, 125-132.

BINGHAM E.T., HURLEY L.V., KAATZ D.M. & SAUNDERS J.W. 1975 Breeding alfalfa which regenerates from callus tissue in culture. Crop Sci. 15, 719-721.

BIONDI S. & THORPE T.A. 1982 Growth regulator effects, metabolite changes, and respiration during shoot initiation in cultured cotyledon explants of *Pinus radiata*. Bot. Gaz. 143, 20-25.

BONNETT H.T. & TORREY J.G. 1965 Chemical control of organ formation in root segments of *Convolvulus* cultured *in vitro*. Plant Physiol. 40, 1228-1236.

BOOTH A. & SATCHUTHANANTHAVALE R. 1974 Regeneration in root cutting of *Taraxacum officinale*. I. Effects of exogenous hormones on root segments and root callus cultures. New Phytol. 73, 453-460.

BOURGIN J.-P., CHUPEAU Y. & MISSONIER C. 1979 Plant regeneration from mesophyll protoplasts of several *Nicotiana* species. Physiol. Plant. 45, 288-292.

BOVO O.A., MROGINSKI L.A. & REY H.Y. 1986 Regeneration of plants from callus tissue of the pasture legume *Lotononis bainesii*. Plant Cell Rep. 5, 295-297.

BOWES B.G. 1976a *In vitro* morphogenesis of *Crambe maritima* L. Protoplasma 89, 185-188.

BOWES B.G. 1976b Polar regeneration in excised roots of *T. officinale* Weber. Ann. Bot. 40, 423-432.

BOWLER C. 1997 The transduction of light signals by phytochrome. *in* Aducci P. (ed.) *Signal Transduction in Plants*.. Birkhauser Verlag, Basel.

BRAND M.H. & LINEBERGER R.D. 1986 *In vitro* propagation of *Halesia carolina* L. and the influence of explantation timing on initial shoot proliferation. Plant Cell Tissue Organ Cult. 7, 103- 113.

BRAWLEY S.H., WETHERELL D.F. & ROBINSON K.R. 1984 Electrical polarity in embryos of wild carrot precedes cotyledon differentiation. Proc. Natl. Acad. Sci. U.S.A., 81, 6064-6067.

BRIGGS W. R. & LISCUM E. 1997 Blue light-activated signal transduction in higher plants. *in* Aducci P (ed.).*Signal Transduction in Plants*. Birkhauser Verlag, Basel.

BROERTJES C. 1969 Mutation breeding of *Streptocarpus*. Euphytica, 18, 333-339.

BROERTJES C. 1972a Mutation breeding of *Achimenes*. Euphytica, 21, 48-62.

BROERTJES C. 1972b Use in plant breeding of acute, chronic or fractionated doses of X-rays or fast neutrons as illustrated with leaves of *Saintpaulia*. Thesis Centr. Agric. Publ. Doc., Wageningen Agric. Res. Rep. No. 776.

BROERTJES C. & KEEN A. 1980 Adventitious shoots: do they develop from one cell? Euphytica 29, 73-87.

BROERTJES C. & LEFFRING L. 1972 Mutation breeding of *Kalanchoe*. Euphytica 21, 415-423.

BROERTJES C. & VAN HARTEN A.M. 1978 *Application of Mutation Breeding in the Improvement of Vegetatively Propagated Crops*. Elsevier Scientific Publishing Co. Amsterdam, Oxford, New York. I.S.B.N. 0-444- 41618-8

BROERTJES C., HACCIUS B. & WEIDLICH S. 1968 Adventitious bud formation on isolated leaves and its significance for mutation breeding. Euphytica 17, 321-344.

BROERTJES C., ROEST S. & BOKELMANN G.S. 1976 Mutation breeding of *Chrysanthemum morifolium* using *in vivo* and *in vitro* adventitious bud techniques. Euphytica 25, 11-19.

BROWN D.C.W. & ATANASSOV A. 1985 Role of genetic background in somatic embryogenesis in *Medicago*. Plant Cell Tissue Organ Cult. 4, 111-122.

BROWNING G., OGNJANOV V., PASSEY A.J. & JAMES D.J. 1987 Multiple shoot and root regeneration from pear embryo cotyledon explants *in vitro*. J. Hort. Sci. 62, 305-311.

BUIATTI M., BARONCELLI S. & BENNICI A. 1974b Genetics of growth and differentiation *in vitro* of *Brassica oleracea* var. *botrytis*. IV. Genotype-hormone interactions. Z. Pflanzenzucht. 73, 298-302.

BUIATTI M., BARONCELLI S., BENNICI A., PAGLIAI M. & TESI R. 1974a Genetics of growth and differentiation *in vitro* of *B. oleracea* var. *botrytis*. II. An *in vitro* and *in vivo* analysis of a diallel cross. Z. Pflanzenzucht. 72, 269-274.

BULLOCK W.P., BAENZIGER P.S., SCHAEFFER G.W. & BOTTINO P.J. 1982 Anther culture of wheat (*Triticum aestivum* L.) F1's and their reciprocal crosses. Theor. Appl. Genet. 62, 155-159.

BURGER D.W. & HACKETT W.P. 1986 Gradients of adventitious bud formation on excised epicotyl and root sections of Citrus. Plant Sci. 43, 229-232.

BUTTON J., KOCHBA J. & BORNMAN C.H. 1974 Fine structure of and embryoid development from embryogenic ovular callus of 'Shamouti' orange (*Citrus sinensis* Osb.). J. Exp. Bot. 25, 446-457.

CALLOW P., HAGHIGHI K., GIROUX M. & HANCOCK J. 1988 *In vitro* shoot regeneration on leaf tissue from micropropagated highbush blueberry. HortScience 23, 807-808.

CAMPBELL R.A. & DURZAN D.J. 1978 The potential for cloning white spruce *via* tissue culture. 86 Pest Control Section, FOR. Management Br., Min. Nat. Resources, Maple, Ont. Canada. (PBA 48 10115) .

CANAS L.A. & BENBADIS A. 1988 *In vitro* plant regeneration from cotyledon fragments of the olive tree (*Olea europaea* L.) Plant Science 54, 65-74.

CASSELLS A.C. 1979 The effect of 2,3,5-tri-iodobenzoic acid on caulogenesis in callus cultures of tomato and *Pelargonium*. Physiol. Plant. 46, 159-164.

CHANDLER S.F., DODDS J.H. & HENSHAW G.G. 1982 Factors affecting adventitious shoot formation in *Solanum laciniatum* Ait. callus cultures. pp. 133-134 in Fujiwara A. (ed.) 1982 (q.v.)

CHEAH K.-T. & CHENG T.-Y. 1978 Histological analysis of adventitious bud formation in cultured Douglas fir cotyledon. Am. J. Bot. 65, 845-849.

CHEN Z., EVANS D.A., SHARP W.R., AMMIRATO P.V. & SONDAHL M.R. 1990 *Handbook of Plant Cell Culture Vol. 6. Perennial Crops*. McGraw-Hill Publishing Company. ISBN 0-07-010848-X

CHENG T. & SMITH H.H. 1973 The influence of genomes on autonomous growth of pith cultures of *N. glauca-langsdorffii* hybrids. Planta 113, 29-34.

CHENG T.-Y. 1976a Organogenesis in culture from cotyledon explants of Douglas fir. Plant Physiol. 57, Suppl., 66.

CHENG T.-Y. 1977 Factors effecting adventitious bud formation of cotyledon culture of Douglas fir. Plant Sci. Lett. 9, 179-187.

CHLYAH H. 1974 Inter-tissue correlations in organ fragments: organogenetic capacity of tissues excised from stem segments of *Torenia fournieri* Lind. cultured separately *in vitro*. Plant Physiol. 54, 341-348.

CHRISTIANSON M.L. & WARNICK D.A. 1985 Temporal requirement for phytohormone balance in the control of organogenesis *in vitro*. Dev. Biol. 112, 494-497.

CHRISTIANSON M.L. 1987 Causal effects in morphogenesis. pp. 45-55 *in* Green C.E, Sommers D.A., Hackett W.P. and Biesboer D.D. (eds) *Plant Tissue and Cell Culture*. Alan R. Liss, Inc., New York.

CHUN Y.W., HALL R.B. & STEPHENS L.C. 1986 Influences of medium consistency and shoot density on *in vitro* shoot proliferation of *Populus alba* x *P. grandidentata*. Plant Cell Tissue Organ Cult. 5, 179-185.

COEN E.S. & MEYEROWITZ E.M. 1991 The war of the whorls: genetic interactions controlling flower development. Nature 35, 31-7.

CROUCH M.L. 1982 Non-zygotic embryos of *Brassica napus* L. contain embryo-specific storage proteins. Planta 156, 520-524.

CUMMINGS P.D., GREEN C.E. & STUTHMAN D.D. 1976 Callus induction and plant regeneration in oats. Crop Sci. 16, 465-470.

CUTTER E.G. 1962 Regeneration in *Zamioculcas*: an experimental study. Ann. Bot. 26, 55-70.

DAVID H., DOMON J-M., SAVY C., MIANNAY N., SULMON G., DARGENT R. & DAVID R. 1992 Evidence for early stages of somatic embryo development in a protoplast-derived cell culture of *Prunus avium*. Physiol. Plant. 85 301-310.

DAVIES M.E. & DALE M.M. 1979 Factors affecting *in vitro* shoot regeneration on leaf discs of *Solanum laciniatum*. Z. Pflanzenphysiol. 92, 51-60.

DE KLERK D.J. 1997 Auxin-cytokinin interactions in. higher plants: old problems and new tools. Trends Plant Sci. 2, 351–356.

DE NETTANCOURT D., DIJKHUIS P., VAN GASTEL A.J.G. & BROERTJES C. 1971 The combined use of leaf irradiation and of the adventitious bud technique for inducing and detecting polyploidy, marker mutations and self-incompatibility in clonal populations of *Nicotiana alata* Link & Otto. Euphytica, 20, 508-521.

DENNIS S. & ASCHER P.D. 1976 Tissue culture of bulb scale sections for asexual propagation of *Lilium*. HortScience 11, 311.

DEWITTE. W., CHIAPPETTA A., AZMI A., WITTERS E., REMBUR J., NOIN M., CHRIQUIE D. & VAN OCKELEN H. 1999 Dynamics of cytokinins in apical shoot meristems of a day-neutral tobacco during floral transition and flower formation. Pl. Physiol. 119, 111-121.

DHARMASIRI N.,DHARMASIRI S., & ESTELLE M. 2005. The F-box protein TIR1 is an auxin receptor. Nature 435, 441–445.

DOMONEY C. & TIMMINS J.N. 1980 Ribosomal RNA gene redundancy in juvenile and mature ivy. J. Exp. Bot. 31, 1093-1100.

DOORBENBOS J. & KARPER J.J. 1975 X-ray induced mutations in *Begonia* x *hiemalis*. Euphytica 24, 13-19.

DOR J. 1955 Studies in the regeneration of horseradish. Ann. Bot. 19, 127-137.

DUNSTAN D.I. & SHORT K.C. 1979 Shoot production from the flower head of *Allium cepa* L. Sci. Hortic. 10, 345-356.

DUNWELL J.M. & CORNISH M. 1980 Regeneration of plants from immature embryos of *Secale cereale*. Ann. Rep. John Innes Inst. 1979, 59-60.

DUNWELL J.M. 1981a *In vitro* regeneration from excised leaves of three *Brassica* species. J. Exp. Bot. 32, 789-799.

DUNWELL J.M. 1981b Influence of genotype and environment on growth of barley *Hordeum vulgare* embryos *in vitro*. Ann. Bot. 48, 535-542.

DURZAN D.J. & GUPTA P.K. 1987 Somatic embryogenesis and polyembryogenesis in Douglas fir cell suspension cultures. Plant Sci. 1952, 229-235.

ELENA E.B. & GINZO H.D. 1988 Effect of auxin levels on shoot formation with different embryo tissues from a cultivar and a commercial hybrid of wheat (*Triticum aestivum* L.). J. Plant Physiol. 132, 600-603.

EMERY A.E.H. 1955 The formation of buds on the roots of *Chamaenerion angustifolium* (L.) Scop. Phytomorph. 5, 139-145.

EVANS D. A., SHARP W. R. & FLICK C. E. 1981 Growth and behaviour of cell cultures: embryogenesis and organogenesis. pp. 45-113 in Thorpe T. A. (ed) 1981 q. v.

ESPINASSE A. & LAY C. 1989 Shoot regeneration of callus derived from globular to torpedo embryos from 59 sunflower genotypes. Crop Sci. 29, 201-205.

ESTRUCH J.J., GRANNELL A., HANSEN G., PRISEN E., REDIG P., VAN ONCKELEN H., SCHWARZ-SOMMER Z., SOMER H. & SPENA A. 1993 Floral development and expression of floral homeotic genes are influenced by cytokinins. Plant J. 4: 379-384.

EVERETT N.P., WACH M.J. & ASHWORTH D.J. 1985 Biochemical markers of embryogenesis in tissue cultures of the maize inbred B73. Plant Sci. 41, 133-140.

FALAVIGNA A., HUSSEY G. & HILTON J. 1980 The effect of colchicine on proliferating adventitious shoots in *Allium cepa*. Ann. Rep. John Innes Inst. 1979, 58.

FARI M. & CZAKO M. 1981 Relationship between position and morphogenetic response of pepper hypocotyl explants cultured *in vitro*. Sci. Hortic. 15, 207-213.

FASOLO F., ZIMMERMAN R.H., FORDHAM I. & FALBO L. 1988 Adventitious shoot formation on excised leaves of *in vitro* cultured apples: effects of cytokinin, auxin, and leaf age. HortScience 23, 755.

FISH N. & JONES M.G.K. 1988 A comparison of tissue culture response between related tetraploid and dihaploid *S. tuberosum* genotypes. Plant Cell Tissue Organ Cult. 15, 201-210.

FLINN B.S., WEBB D.T. & NEWCOMB W. 1988 The role of cell clusters and promeristemoids in determination and competence for caulogenesis by *Pinus strobus* cotyledons *in vitro*. Can. J. Bot. 66, 1556-1565.

FORCHE E., KIBLER R. & NEUMANN K.-H. 1981 The influence of developmental stages of haploid and diploid callus cultures of *Datura innoxia* on shoot initiation. Z. Pflanzenphysiol. 101, 257-262.

FOROUGHI-WEHR B., FRIEDT W. & WENZEL G. 1982 On the genetic improvement of androgenetic haploid formation in *Hordeum vulgare* L. Theor. Appl. Genet. 62, 233-239.

FRAGA.M.F., CAÑAL M.J. & RODIGUEZ R. 2002a Phase-change related epigenetic and physiological changes in *Pinus radiata* D. Don. Planta 215, 672-678.

FRAGA M. F., CAÑAL M. J. & RODIGUEZ R. 2002b *In vitro* morphogenic potential ofdifferently aged *Pinus radiata* D. Don. trees correlated with polyamines and DNA methylation levels. Plant Cell Tissue Organ Cult. 70, 139-145.

FRAGA M. F., RODRIGUEZ R. & CAÑAL M.J. 2002c Genomic DNA methylation-demethylation during ageing-invigoration of *Pinus radiata* .Tree Physiol. 22. 813-816.

FRANCIS D., DUDITS D. & INZE D. 1998 (eds.) *Plant Cell Division*. Research Monograph, Portland Press, London.

FRANKENBERGER E.A. & TIGCHELAAR E.C. 1980 Genetic analysis of shoot formation from excised leaf discs of tomato. In Vitro 16, 217.

FRANKENBERGER E.A., HASEGAWA P.W. & TIGCHELAAR E.C. 1981 Influence of environment and developmental state on the shoot-forming capacity of tomato genotypes. Z. Pflanzenphysiol. 102, 221-232.

FUJIMURA T. & KOMAMINE A. 1980 The serial observation of embryogenesis in a carrot cell suspension culture. New Phytol. 86, 213-218.

FUJIWARA A. (ed.) 1982 *Plant Tissue Culture* 1982. Proc. 5th. Int. Cong. Plant Tiss. Cell Cult., Japan. Assoc. Plant Tissue Culture, Japan.

GABRYSZEWSKA E. & SANIEWSKI M. 1983 Auxin control of tulip stalk elongation *in vitro*. Sci. Hortic. 19, 153-159.

GAHAN P.B. 2004 The cell cycle, competence and recalcitrance. Proceedings COST 843 WG 1 Oviedo 29-31.1.04.

GAHAN P.B. & BELLANI L.M. 1984 Identification of shoot apical meristem cells committed to form vascular elements in *Pisum sativum* L and *Vicia faba* L. Ann. Bot. 54, 837-841.

GAHAN P.B. & CARMIGNAC D.F. 1989 Determination of vascular tissue in roots of dicotyledonous plants. pp. 25-28 in Lough-man B.C., Gasparikova O. and J. Kolek J. *Structural and Functional Aspects of Roots*. Kluwer Acad. Publishing Co., London.

GAHAN P.B., MCGARRY A., WANG, L. DORÉ T. & CARMIGNAC D.F. 1996 Glucose-6-phosphate and UDP-D-glucose dehydrogenases: possible markers of vascular differentiation. Phytochem. Analysis 8, 110-114.

GAHAN P.B., PINTO E., COURT S., EZE E. K., WANG L. & MANTELL S.H. 1994 Plant growth regulator induced xylogenesis in cotyledons of *Solanum aviculare*. J. Exp. Bot. 45, 1523-1532.

GAHAN P.B. & RANA M.A. 1985 The quiescent centre and cell determination in roots of *Pisum sativum*. Ann. Bot. *56*, 438-442.

GAHAN P.B., WANG L. & WYNDAELE,R. 1995 Possible mechanisms of plant bioregulator modulation of mesophyll cells to form vessels. Proc. 9th Forum for Applied Biotechnology, Sept 1995, 27-29.

GAHAN P.B. 1981 An early cytochemical marker of commitment to stellar differentiation in meristems from dicotyledonous plants. Ann. Bot. 48, 769-775.

GAHAN P.B. 2004 Messenger DNA in higher plants. Cell Biochem. Funct. 21, 207-209.

GAHAN P.B. 2006 Totipotency and the cell cycle. *in* Mohan Jain S. and Häggman H. (eds.) *Protocols for Micropropagation of Woody Trees and Fruits*. Springer, The Netherlands

GEIER T. & SANGWAN R. S. 1996 Histology and chimeral segregation reveal cell-specific differences in the competence for shoot regeneration and *Agrobacterium*-mediated transformation in *Kohleria* internode explants. Pl. Cell Rep. 15 386-390.

GHARYAL P.K. & MAHESHWARI S.C. 1981 *In vitro* differentiation of somatic embryoids in a leguminous tree - *Albizzia lebeck* L. Naturwissenschaften. 68, 379-380.

GOSCH-WACKERLE G., AVIVI L. & GALUN E. 1979 Induction, culture and differentiation of callus from immature rachises, seeds and embryos of *Triticum*. Z. Pflanzenphysiol. 91, 267-278.

GOTO N. 1981 Organogenetic capacity of mutant lines and their hybrids in *Arabidopsis thaliana*. Bot. Mag. Tokyo 94, 77-82.

GOULD A.R. 1978 Diverse pathways of morphogenesis in tissue cultures of the composite *Brachycome lineariloba* (2n = 4). Protoplasma 97, 125-135.

GRECKA K. 1987 *In vitro* propagation of horse-radish (*Cochlearia armoracia* L.). Acta Hortic. 212, 671-674.

GRECO B., TANZARELLA O.A., CARROZZO G. & BLANCO A. 1984 Callus induction and shoot regeneration in sunflower (*Helianthus annuus*). Plant Sci. Lett. 36, 73-77.

GREEN C.E. & PHILLIPS R.L. 1975 Plant regeneration from tissue cultures of maize. Crop Sci. 15, 417-427.

GRESSHOFF P.M. & DOY C.H. 1972 Haploid *Arabidopsis thaliana* callus and plants from anther culture. Aust. J. Biol. Sci. 25, 259-264.

GREWAL S., KOUL S., SACHDEVA U. & ATAL C.K. 1977 Regeneration of plants of *Dioscorea deltoides* Wall. by apical meristem cultures. Ind. J. Exp. Biol. 15, 201-203.

GUERRA M.P. & HANDRO W. 1988 Somatic embryogenesis and plant regeneration in embryo cultures of *Euterpe edulis* Mart. (*Palmae*). Plant Cell Rep. 7, 550-552.

GUNAY A.L. & RAO A.S. 1978 *In vitro* plant regeneration from hypocotyl and cotyledon explants of red pepper. Plant Sci. Lett. 11, 365-372.

GUPTA P.K. & DURZAN D.J. 1986 Somatic embryogenesis from callus of mature sugar pine embryos. Nat. Biotechnol. 4, 643-645.

GUPTA P.K., DANDEKAR A.M. & DURZAN D.J. 1988 Somatic proembryo formation and transient expression of a luciferase gene in Douglas fir and loblolly pine protoplasts. Plant Sci. 58, 85-92.

HACCIUS B. 1978 Question of unicellular origin of non-zygotic embryos in callus cultures. Phytomorph. 28, 74-81.

HACKETT W.P. 1969 Aseptic multiplication of lily bulblets from bulb scales. Comb. Proc. Int. Plant Prop. Soc. 19, 105-108.

HAHNE G., MAYER J.E. & LÖRZ H. 1988 Embryogenic and callus-specific proteins in somatic embryogenesis of the grass *Dactylis glomerata* L. Plant Sci. 55, 267-279.

HALL M. A. & SMITH A.R. 1997 Transduction and ethylene response. *in* Aducci P. (ed.) *Signal Transduction in Plants*. Birkhauser Verlag, Basel.

HALPERIN W. 1966 Alternative morphogenetic events in cell suspensions. Am. J. Bot. 53, 443-453.

HALPERIN W. 1967 Population density effects on embryogenesis in carrot cell cultures. Exp. Cell Res. 48, 170-173.

HAMILTON A., VOINNET O,. CHAPPELL L. & BAULCOMBE D. 2002 Two classes of short interfering RNA in RNA silencing. EMBO J. 21, 4671-4769.

HAMMATT N., NELSON R.S. & DAVEY M.R. 1987 Plant regeneration from seedling explants of perennial *Glycine* species. Plant Cell Tissue Organ Cult. 11, 3-11.

HAMMERSCHLAG F. & BOTTINO P. 1981 Effect of plant age on callus growth, plant regeneration and anther culture of geranium. J. Am. Soc. Hort. Sci. 106, 114-116.

HAMMERSCHLAG F. 1982 Factors affecting establishment and growth of peach shoots *in vitro*. HortScience 17, 85-86.

HANSON J. & READ P.E. 1981 A comparison of *in vitro* culture and whole plant performance of four *Petunia hybrida* cultivars and their inbred parents. HortScience 16, 426.

HANSON J.B. & TREWAVAS A.J. 1982 Regulation of plant cell growth: the changing perspective. New Phytol. 90, 1-18.

HARI V. 1980 Effect of cell density changes and conditioned media on carrot cell embryogenesis. Z. Pflanzenphysiol. 96, 227-231.

HARNEY P.M. & KNAP A. 1979 A technique for the *in vitro* propagation of African violets using petioles. Can. J. Plant Sci. 59, 263-266.

HE G.G., TANNER G. & SCOTT K.J. 1986 Somatic embryogenesis and morphogenesis in callus derived from the epiblast of immature embryos of wheat (*Triticum aestivum*). Plant Sci. 45, 119-124.

HENKE R.R., HUGHES, K.W., CONSTANTIN M.J., HOLLAENDER A.A. & WILSON C.M. (eds.) 1985 *Tissue Culture in Forestry and Agriculture*. Plenum Press, New York, London.

HEPHER A., BOULTER M.E., HARRIS N. & NELSON R.S. 1988 Development of a superficial meristem during somatic

embryogenesis from immature cotyledons of soybean (*Glycine max* L.). Ann. Bot. 62, 513-519.

HICKS G.S. 1980 Patterns of organ development in plant tissue culture and the problem of organ determination. Bot. Rev. 46, 1-23

HOBBIE, L.J. 2003 Auxin pp.186-197 in Henrey H.L. & Norman A.W.(eds.) *Encyclopedia of Hormones. I.* Academic Press, NewYork.

HONMA T. & GOTO K. 2001 Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature 409, 525-529.

HWANG L.S., SKIRVIN R.M. & BOUWKAMP J. 1981 Shoot formation from sweet potato (*Ipomoea batatas* Lam.) tuberous discs *in vitro*. HortScience 16, 433.

INOUE T, HIGUCHI M, HASHIMOTO Y, SEKI M. KOBA-YASHI M. & KATO T. 2001 Identification of CRE1 as a cyto-kinin receptor from *Arabidopsis*. Science 409, 1060-1063.

IZHAR S. & POWER J.B. 1977 Genetical studies with Petunia leaf protoplasts. 1. Genetic variation to specific growth hormones and possible genetic control on stages of protoplast development in culture. Plant Sci. Lett. 8, 375-383.

JAGADISH CHANDRA K.S. & SREENATH H.L. 1982 *In vitro* culture and morphogenetic studies in some species of *Cymbopogon* Spreng. (aromatic grasses). pp. 703-705 in Fujiwara (ed.) 1982 (q.v.).

JAISWAL S.K., BHOJWANI S.S. & BHATNAGAR S.P. 1987 *In vitro* regeneration potentialities of seedling explants of *Brassica carinata* A. Braun. Phytomorph. 37, 235-241.

JANSSON E. & BORNMAN C.H. 1981 *In vitro* initiation of adventitious structures in relation to the abscission zone in needle explants of *Picea abies*: anatomical considerations. Physiol. Plant. 53, 191-197.

JELASKA S. & JELENCIC B. 1980 Plantlet regeneration from shoot tip culture of *Pelargonium zonale* hybrid. Acta Bot. Croat. 39, 59-64.

JONES H.D., SMITH S.J., DESIKAN R., PLAKIDOU-DYMOCK S. & LOVEGROVE A. 1998 Heterotrimeric G proteins are implicated in gibberellin induction of α-amylase in wild oat aleurone. Plant Cell, 10, 245–253.

JONES T.J. & ROST T.L. 1989 The developmental anatomy and ultrastructure of somatic embryos from rice (*Oryza sativa* L.) scutellum epithelial cells. Bot. Gaz. 150, 41-49.

KADKADE P.G. & JOPSON H.A. 1977 *In vitro* studies of adventitious bud formation in Douglas fir and pine embryo cultures. Plant Physiol. 59, Suppl., 62.

KAKIMOTO T. 1996 CKI1, a histidine kinase homolog impli-cated in cytokinin signal transduction. Science 274, 982-985.

KAMISAKA S. & SHIBATA K. 1977 Anticotyledon factor: competitive inhibitors of the action of dihydroconiferyl alcohol in stimulating gibberellic acid-induced lettuce hypocotyl elongation. Plant Cell Physiol. 18, 1057-1066.

KARTHA K.K., GAMBORG O.L. & CONSTABEL F. 1974 *In vitro* plant formation from stem explants of rape (*Brassica napus* cv. Zephyr). Physiol. Plant. 31, 217-220.

KATHAL R., BHATNAGAR S.P. & BHOJWANI S.S. 1988 Regeneration of plants from leaf explants of *Cucumis melo* cv. Pusa Shaibati. Plant Cell Rep. 7, 449-451.

KATO Y. 1974 Bud formation on excised *Heloniopsis* leaf fragments: effects of leaf age and the midrib. Plant Cell Physiol. 15, 363-372.

KATO Y. 1976 Induced budding in root-tips and attached leaves of *Heloniopsis orientalis* (Liliaceae). Plant Sci. Lett. 6, 431-436.

KATO Y. 1978 Induction of adventitious buds on undetached leaves, excised leaves, and leaf fragments of *Heloniopsis orientalis*. Physiol. Plant. 42, 39-44.

KAUL K. 1985 Seasonal variation in callus proliferation from explants of mature *Pinus strobus* trees. p. 330 in Henke *et al.*, (eds.) 1985 (q.v.).

KAUL K. 1986 Establishment of long-term callus cultures from mature white pine (*Pinus strobus*, Pinaceae). Am. J. Bot. 73, 242-245.

KAVI KISHOR P.B. & REDDY G.M. 1986 Regeneration of plants from long-term cultures of *Oryza sativa* L. Plant Cell Rep. 5, 391-393.

KAWASAKI H & TAIRA K. 2004 Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. Nature 431, 211-216.

KEPINSKI S. & LEYSER O. 2005. The *Arabidopsis* F-box protein TIR1 is an auxin receptor. Nature 435, 446–451.

KEYES G.J. & BINGHAM E.T. 1979 Heterosis and ploidy effects on the growth of alfalfa callus. Crop Sci. 19, 473-476.

KEYES G.J., DEATON W.R., COLLINS G.B. & LEGG P.D. 1981 Hybrid vigour in callus tissue cultures and seedlings of *Nicotiana tabacum* L. J. Hered. 72, 172-174.

KHALID N., DAVEY M.R. & POWER J.B. 1989 An assessment of somaclonal variation in *Chrysanthemum morifolium*: the generation of plants of potential commercial value. Sci. Hortic. 38, 287-294.

KHAN M.R.I., HEYES J.K. & COHEN D. 1988 Plant regeneration from oca (*Oxalis tuberosa* M.): The effect of explant type and culture media. Plant Cell Tissue Organ Cult. 14, 41-50.

KHODER M., VILLEMUR P. & JONARD R. 1979 Micro-propagation et bouturage *in vitro* chez le jasmin (*Jasminum officinale* L.). Compt. Rend. Acad. Sci. Paris 288D, 323-326.

KIM S.-G., CHANG J.-R., CHA H.C. & LEE K.-W. 1988 Callus growth and plant regeneration in diverse cultivars of cucumber (*Cucumis sativus* L.). Plant Cell Tissue Organ Cult. 12, 67-84.

KLIMASZEWSKA K. 1979 The regenerative potential of two species of *Peperomia* in tissue cultures and biological activity of endogenous growth regulators. Acta Hortic. 91, 281-286.

KOEVARY K., RAPPAPORT L. & MORRIS L.L. 1978 Tissue culture propagation of head lettuce. HortScience 13, 39-41.

KOHLENBACH H.W. 1977 Basic aspects of differentiation and plant regeneration from cell and tissue cultures. pp.355-366 in Barz W., Reinhard E. & Zenk M.H. (eds.) 1977 *Plant Tissue Culture and its Biotechnological Application.* Springer-Verlag, Berlin, Heidelberg, New York.

KONAR R.N., THOMAS E. & STREET H.E. 1972 Origin and structure of embryoids arising from epidermal cells of the stem of *Ranunculus sceleratus* L. J. Cell Sci. 11, 77-93.

KORBAN S.S. & SKIRVIN R.M. 1985 *In vitro* shoot regeneration from an intact and a sectioned embryo-axis of apple seeds. Plant Sci. 39, 61-66.

KOUADIO J.Y. & PHAN C.T. 1987 Heterogeneity of progeny in meristem culture. Acta Hortic. 212, 411-417.

KOUIDER M., KORBAN S.S., SKIRVIN R.M. & YOUNG H. 1985 The relationship of apple embryos and their cotyledons to maturity, dormancy, and the potential to form shoots *in vitro*. J. Am. Soc. Hort. Sci. 110, 93-96.

KROGSTRUP P., ERIKSEN E.N., MLLER J.D. & ROULUND H. 1988 Somatic embryogenesis in Sitka spruce (*Picea sitchensis* Bong. Carr.). Plant Cell Rep. 7, 594-597.

KUIJPERS A.-.M. & LANGENS-GERRITS M. 1997 Propaga-tion of tulip *in vitro*. Acta Hortic. 430: 321-332.

KURZ M.L. 1986 Tissue culture of conifers. Comb. Proc. Int. Plant Prop. Soc. 35, 206-216.

LA RUE C.D. 1954 p. 187 in Brookhaven Symp. Biol. 6. Brookhaven National Laboratory, New York.

LALL S, NETTLETON D, DECOOK R, CHE P. & HOWELL S.H. 2004 Quantitative trait loci associated with adventitious shoot formation in tissue culture and the program of shoot development in *Arabidopsis*. Genetics 167, 1883-92.

LAZAR M.D., BAENZIGER P.S. & SCHAEFFER G.W. 1984 Combined abilities and heritability of callus formation and plantlet regeneration in wheat (*Triticum aestivum* L.) anther cultures. Theor. Appl. Genet. 68, 131-134.

LAZAR M.D., COLLINS G.B. & VIAN W.E. 1983 Genetic and environmental effects on the growth and differentiation of wheat somatic cell cultures. J. Hered. 74, 353-357.

LAZZERI P.A. & DUNWELL J.M. 1986 *In vitro* regeneration from seedling organs of *Brassica oleracea* var. *italica* cv. Green Comet. II. Effect of light conditions and explant size. Ann. Bot. 58, 699-710.

LEGRAND B. 1972 Influence des conditions initiales d'éclairement sur le bourgeonnement et la polarité de tissus de feuilles d'endive cultivés *in vitro*. Compt. Rend. Acad. Sci. Paris 275D, 31-32.

LEIBFREID A., TO J.P.C., BUSCH W., STEHLING S., KEHLE A., DEMAR M., KEIBER J.J. & LOHMANN, J.U. 2005 WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature 438, 1172-1175.

LESHEM B., LILIEN-KIPNIS H. & STEINITZ B. 1982 The effect of light and of explant orientation on the regeneration and subsequent growth of bulblets on *Lilium longiflorum* Thunb. bulb-scale sections cultured *in vitro*. Sci. Hortic. 17, 129-136.

LINSMAIER E.M. & SKOOG F. 1965 Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18, 100-127.

LIPMANN Z. & MARTIENNSEN R. 2004 The role of RNA inter ferencein heterochromatic silencing. Nature 431, 364-370.

LIU J.R. & CANTLIFFE D.J. 1984 Somatic embryogenesis and plant regeneration in tissue cultures of sweet potato (*Ipomoea batatas* Poir.). Plant Cell Rep. 3, 112-115.

LIU L. & BURGER D.W. 1986 *In vitro* propagation of Easter lily from pedicels. HortScience 21, 1437-1438.

MAHESWARAN G. & WILLIAMS E.G. 1985 Origin and development of somatic embryoids formed directly on immature embryos of *Trifolium repens in vitro*. Ann. Bot. 56, 619-630.

MADSEN M., FREDERIKSEN C. G., NAUERBY, B. & WYNDAELE, R. 1994 Transformation potential of two transformation systems for pea (*Pisum sativum*). pp.63-78 in Ph.D. thesis, University of Aarhus, Århus, Denmark.

MANDAL S. & GADGIL V.N. 1979 Differentiation of shoot buds and roots in fruit callus of *Solanum nigrum* L. Ind. J. Exp. Biol., 17, 876-878.

MANGAT B.S. & ROY M.K. 1986 Tissue culture and plant regeneration of okra (*Abelmoschus esculentus*). Plant Sci. 47, 57-61.

MARGARA J. & LEYDECKER M.-T. 1978 Différents types d'organogenèse observés chez le Colza, *Brassica napus* L. var. *oleifera* Metzg. Compt. Rend. Acad. Sci. Paris 287D, 17-20.

MARGARA J. 1969 Etude preliminaire des facteurs de la néoformations de bourgeons chez le chou-fleur (*Brassica oleracea* L. var. *botrytis*). Compt. Rend. Acad. Sci. Paris 268D, 686-688.

MARGARA J. 1977 La multiplication végétative de la betterave (*Beta vulgaris* L.) en culture *in vitro*. Compt. Rend. Acad. Sci. Paris 285D, 1041-1044.

MARIOTTI D. & ARCIONI S. 1983 *Coronilla varia* L. (Crown vetch): plant regeneration through somatic embryogenesis. Plant Cell Tissue Organ Cult. 2, 103-110.

MARKS T. R. 1998 A simple internode system for studying the development of rhizogenesis. pp. 36-37 in *Physiology and Control of Plant Propagation*. Proc. COST 822 (1996), Berlin.

MARKS T. R. & SIMPSON S. E. 2000 Rhizogenesis in *Forsythia* x *intermedia* and *Syringia vulgaris*; application of asimpl internode experimental system. Plant Cell Rep. 19, 1171-1176.

MATHEWS H. 1987 Morphogenetic responses from *in vitro* cultured seedling explants of mung bean (*Vigna radiata* L. Wilczek). Plant Cell Tissue Organ Cult. 11, 233-240.

MATHIAS R.J. & FUKUI K. 1986 The effect of specific chromosome and cytoplasmic substitutions on the tissue culture response of wheat (*Triticum aestivum*) callus. Theor. Appl. Genet. 71, 797-800.

MATHIAS R.J., FUKUI K. & LAW C.N. 1986 Cytoplasmic effects on the tissue culture reponse of wheat (*Triticum aestivum*) callus. Theor. Appl. Genet. 72, 70-75.

MATSUBARA S. & CLORE W.J. 1974 Vegetative propagation of asparagus from lateral buds. Sci. Rep., Fac. Agr., Okayama Univ., Jap. 43, 19-26.

MATSUBARA S. 1973 Population effect in lateral bud culture of asparagus and promotion of root formation by transplanting. J. Jap. Soc. Hort. Sci. 42, 142-146.

MATSUOKA H. & HINATA K. 1979 NAA-induced organogenesis and embryogenesis in hypocotyl callus of *Solanum melongena* L. J. Exp. Bot. 30, 363-370.

MATZKE M.A. & MATZKE A.J.M. 2000 *Plant Gene Silencing*. Kluwer, Dordrecht.

MAUSETH J.D. & HALPERIN W. 1975 Hormonal control of organogenesis in *Opuntia polyacantha* (Cactaceae). Am. J. Bot. 62, 869-877.

MCCABE P.F., LEVINE A., MEIJER P.J., TAPON N.A. & PENEL R. I. 1977 A density programmed cell death pathway activated in carrot cells cultured at low density. Plant J. 12, 267-280.

McCOMB J.A. & BENNETT I.J. 1982 Vegetative propagation of *Eucalyptus* using tissue culture and its application to forest improvement in Western Australia. pp. 721-722 in Fujiwara (ed.) 1982 (q.v.).

McCOY T.J. & BINGHAM E.T. 1977 Regeneration of diploid alfalfa plants from cells grown in suspension culture. Plant Sci. Lett. 10, 59-66.

McCOY T.J. & BINGHAM E.T. 1979 Breeding and selection of diploid alfalfa (*Medicaga sativa* L.) that regenerates plants from cells grown in suspension culture. p. 851 in Sharp *et al.,* (eds.) 1979 (q.v.).

MACDONALD H. 1997 Auxin perception and signal transduction. Physiol. Plant. 100, 423-430.

MCGARRY A. 1988 A cytochemical investigation of cytodifferentiation in root apices of *Pisum sativum* var. Meteor and calluses of *Malus pumilla* Mill. Ph.D. thesis, University of London.

McWILLIAM A.A., SMITH S.M. & STREET H.E. 1974 The origin and development of embryoids in suspension cultures of carrot (*Daucus carota*). Ann. Bot. 38, 243-250.

MEDEROS S. & RODRGUEZ ENRQUEZ M.J. 1987 *In vitro* propagation of 'Golden Times' roses. Factors affecting shoot tips and axillary bud growth and morphogenesis. Acta Hortic. 212, 619-624.

MEHRA P.N. & JAIDKA K. 1985 Experimental induction of embryogenesis in pear. Phytomorph. 35, 1-10.

MIKAMI T. & KINOSHITA T. 1988 Genotypic effects on the callus formation from different explants of rice *Oryza sativa* L. Plant Cell Tissue Organ Cult. 12, 311-314.

MIKKELSEN S.P. & SINK K.C. 1978 Histology of adventitious shoot and root formation on leaf-petiole cuttings of *Begonia* x *hiemalis* Forsch 'Aphrodite Peach'. Scientia Hort. 8, 179-192.

MIURA Y. & TABATA M. 1986 Direct somatic embryogenesis from protoplasts of *Foeniculum vulgare*. Plant Cell Rep. 5, 310-313.

MOHAPATRA D. & BAJAJ Y.P.S. 1988 Hybridization in *Brassica juncea* x *Brassica campestris* through ovary culture. Euphytica 37, 83-88.

MOSHKOV I.E. NOVIKOVA G.V., MUR L. A.J., SMITH A.R. & HALL M.A. 2003a Ethylene regulates monomeric GTP-binding protein gene expression and activity in *Arabidopsis*. Plant Physiol. 131, 1705–1717.

MOSHKOV I.E., NOVIKOVA G.V., MUR L.A.J., SMITH A.R & HALL M.A. 2003b Ethylene rapidly up-regulates the activities of both monomeric GTP-binding proteins and protein kinase(s) in epicotyls of pea. Plant Physiol. 131, 1718-1726.

MURASHIGE T. & TUCKER D.P.H. 1969 Growth factor requirements of citrus tissue culture. pp. 1155-1161 in Chapman H. D. (ed.) Proc. 1st Int. Citrus Symp. Vol. 3, Univ. Calif., Riverside Publication.

MURRAY R.E., HANDYSIDE R.L. & KELLER W.A. 1977 *In vitro* regeneration of shoots on stem explants of haploid and diploid flax (*Linum usitatissimum*). Can J. Genet. Cytol. 19, 177-186.

NAG K.K. & JOHRI B.M. 1976 Experimental morphogenesis of the embryo of *Dendrophthoe*, *Taxillus* and *Nuytsia*. Bot. Gaz. 137, 378-390.

NARASIMHULU S.B. & CHOPRA V.L. 1988 Species specific shoot regeneration response of cotyledonary explants of brassicas. Plant Cell Rep. 7, 104-106.

NARASIMHULU S.B., PRAKESH S. & CHOPRA V.L. 1988 Comparative shoot regeneration responses of diploid brassicas and their synthetic amphidiploid products. Plant Cell Rep. 7, 525-527.

NAUERBY B., MADSEN M., CHRISTIANSON J. & WYNDAELE R. 1990 Regeneration of transgenic pea plants from thin cell layer segments. Abstr. VII Int. Congr. on Plant Tissue and Cell Culture. Amsterdam 24-29 June 1990 p53

NAUERBY B., MADSEN M., CHRISTIANSON J. & WYNDAELE R. 1991 A rapid and efficient regeneration system for pea (*Pisum sativum*), suitable for transformation. Plant Cell Rep. 9, 676-679.

NEGRUTIU I. & JACOBS M. 1978 Factors which enhance *in vitro* morphogenesis of *Arabidopsis thaliana*. Z. Pflanzenphysiol. 90, 423-430.

NEHRA N.S., STUSHNOFF C. & KARTHA K.K. 1988 Direct shoot regeneration from leaf discs in strawberry. HortScience 23, 808.

NIEDERWIESER J.G. & VCELAR B.M. 1990 Regeneration of *Lachenalia* species from leaf explants. HortScience 25, 684-687.

NIIMI Y. & ONOZAWA T. 1979 *In vitro* bulblet formation from leaf segments of lilies, especially *Lilium rubellum*. Sci. Hortic. 11, 379-390.

NOBUHARU G. 1980 Organogenetic capacity and its heritability. *Arabidopsis* Inf. Serv. 0 (17), 49-58 (BA 71, 6607)

NOGUE F., GONNEAU M. & FAURFE J-D. 2003 Cytokinins pp. 371-378 in Henrey H.L. & Norman A.W.(eds.) *Encyclopedia of Hormones. I.* Academic Press, NewYork.

NOMURA K. & KOMAMINE A. 1985 Identification and isolation of single cells that produce somatic embryos at a high frequency in a carrot suspension culture. Plant Physiol. 79, 988-991.

NOMURA K. & KOMAMINE A. 1986 Polarized DNA synthesis and cell division in cell clusters during somatic embryogenesis from single carrot cells. New Phytol. 104, 25-32.

NORIZAKU T., TANIMOTO S. & HARADA H. 1985 Effects of wounding on adventitous bud formation in *Torenia fournieri* stem segments cultured *in vitro*. J Exp. Bot. 36, 841-847.

NORRIS R.E. & SMITH R.H. 1981 Regeneration of variegated African violet (*Saintpaulia ionantha* Wendl.) leaf chimeras in culture. Plant Physiol. 67, Suppl., 117.

NORTHCOTE D.H. 1979 Biochemical mechanisms involved in plant morphogenesis. pp. 11-20 in George E.F. (ed.) 1979 *Differentiation and the Control of Development in Plants – Potential for Chemical Modification.* British Plant Growth Reg. Group, Monograph No. 3, ISBN 0-906673-01-0

NOVIKOVA G.V., MOSHKOV I.E., SMITH A.R. & HALL M.A. 2000 The effect of ethylene on MAPKinase-like activity in *Arabidopsis thaliana*. FEBS Lett. 474, 29-32.

NOVINA C. D. & SHARP P. A. 2004 The RNAi revolution. Nature 430, 161-164.

NOVÁK F.J. & VYSKOT B. 1975 Karyology of callus cultures from *Nicotiana tabacum* L. haploids. Z. Pflanzenzucht. 75, 62-70.

NUTI RONCHI V., NOZZOLINI M. & AVANZI L. 1981 Chromosomal variation on plants regenerated from two *Nicotiana* spp. Protoplasma 109, 433-444.

OGURA H. & TSUJI S. 1977 Differential responses of *Nicotiana tabacum* L. and its putative progenitors to de- and re-differentiation. Z. Pflanzenphysiol. 83, 419-426.

OHKI S., BIGOT C. & MOUSSEAU J. 1978 Analysis of shoot-forming capacity *in vitro* in two lines of tomato (*Lycopersicon esculentum* Mill.) and their hybrids. Plant Cell Physiol. 19, 27-42.

OLESEN P.O. 1978 On cyclophysis and topophysis. Silvae Genet. 27, 173-178.

ORTON T.J. 1980 Chromosome variability in tissue cultures and regenerated plants of *Hordeum*. Theor. Appl. Genet. 56, 101-112.

PAEK K.Y. 1982 Studies on the clonal propagation by the culture of bulb scale segments, inflorescence stems and flower buds in hyacinth. pp. 713-714 in Fujiwara A. (ed.) 1982 (q.v.).

PARK Y.G. & SON S.H. 1988 *In vitro* organogenesis and somatic embryogenesis from punctured leaf of *Populus nigra* x *P. maximowiezii*. Plant Cell Tissue Organ Cult. 15, 95-105.

PELLISIER T. & WASSENEGER M. 2000 A DNA target of 30bp is sufficient for RNA-directed DNA methylation. RNA 6, 55-65.

PHILLIPS G.C. & COLLINS G.B. 1980 Somatic embryogenesis from cell suspension cultures of red clover. Crop Sci. 20, 323-326.

PHILLIPS R. & DODDS J.H. 1977 Rapid differentiation of tracheary elements in cultured explants of Jerusalem artichoke. Planta 135, 207-212.

PIERIK R.L.M. & STEEGMANS H.H. 1972 The effect of 6-benzylamino-purine on growth and development of *Cattleya* seedlings grown from unripe seeds. Z. Pflanzenphysiol. 68, 228-234.

PIERIK R.L.M. & STEEGMANS H.H.M. 1975 Analysis of adventitious root formation in isolated stem explants of *Rhododendron*. Sci. Hortic. 3, 1-20.

PIERIK R.L.M. 1969 Factors affecting adventitious root formation in isolated stem segments of *Rhododendron*. Neth. J. Agric. Sci. 17, 203-208.

PIERIK R.L.M. 1987 Plant Cell Culture Technology - a book review. Sci. Hortic. 33, 308-309.

PLAKIDOU-DYMOCK S., DYMOCK D. & HOOLEY R. 1998 A higher plant seven-transmembrane receptor that influences sensitivity to cytokinins. Current Biol. 8, 315-324.

RADOJEVIC L., SOKIC O. & TUCIC B. 1987 Somatic embryogenesis in tissue culture of iris (*Iris pumila* L.). Acta Hortic. 212, 719-723.

RAGHAVA RAM N.V. & NABORS M.W. 1985 Plant regeneration from tissue cultures of Pokkali rice is promoted by

optimizing callus to medium volume ratio and by a medium-conditioning factor produced by embryogenic callus. Plant Cell Tissue Organ Cult. 4, 241-248.

RAJU M.V.S. & MANN H.E. 1970 Regenerative studies on the detached leaves of *Echeveria elegans*. Anatomy and regeneration of leaves in sterile culture. Can. J. Bot. 48, 1887-1891.

RAJU M.V.S. & MANN H.E. 1971 Regeneration studies on the detached leaves of *Echeveria elegans*. Patterns of regeneration of leaves in sterile culture. Can. J. Bot. 49, 2015-2021.

RANA M.A. & GAHAN P.B. 1982 Determination of stelar elements in roots of *Pisum sativum* L. Ann. Bot. 50, 757-762.

RANA M.A. & GAHAN P.B. 1983 Quantitative cytochemical study of determination for xylem-element formation in response to wounding in roots of *Pisum sativum.* Planta *157,* 307-316.

RANGA SWAMY N.S. 1958 Culture of nucellar tissue of *Citrus in vitro*. Experientia 14, 111-112.

RANGA SWAMY N.S. 1961 Experimental studies on female reproductive structures of *Citrus microcarpa* Bunge. Phytomorph. 11, 109-127.

RANGASWAMY N.S. 1982 Nucellus as an experimental system in basic and applied tissue culture research. pp. 269-286 in Rao A.N. (ed.) 1982 *Tissue Culture of Economically Important Plants*. Proc. Int. Symp., Singapore, 1981. COSTED and ANBS Publications.

RAO P.S., HANDRO W. & HARADA H. 1973 Hormonal control of differentiation of shoots, roots and embryos in leaf and stem cultures of *Petunia inflata* and *P. hybrida*. Physiol. Plant. 28, 458-463.

RAVEN J.A. 1979 The possible role of membrane electrophoresis in the polar transport of IAA and other solutes in plant tissues. New Phytol. 82, 285-291.

REDENBAUGH K., PAASCH B.D., NICHOL J.W., KOSSLER M.E., VISS P.R. & WALKER K.A. 1986 Somatic seeds: encapsulation of asexual plant embryos. Nat. Biotechnol. 4, 797-801.

REDENBAUGH K., SLADE D., VISS P.R. & FUJII J.A. 1987 Encapsulation of somatic embryos in synthetic seed coats. HortScience 22, 803-809.

REDIG P, SHAUL O, INZÉ D, VAN MONTAGU M, VAN ONCKELEN H. 1996 Levels of endogenous cytokinins, indole-3-acetic acid and abscisic acid during the cell cycle of synchronized tobacco BY-2 cells. FEBS Lett. 391, 175-80.

REINERT J. & BACKS D. 1968 Control of totipotency in plant cells growing *in vitro*. Nature 220, 1340-1341.

REINERT J., BACKS-HSEMANN D. & ZERMAN H. 1971 Determination of embryo and root formation in tissue cultures from *Daucus carota*. pp. 261-268 in *Les Cultures de Tissus de Plantes*. Coll. Int. CNRS 193 Paris.

REISCH B. & BINGHAM E.T. 1980 The genetic control of bud formation from callus cultures of diploid alfalfa. Plant Sci. Lett. 20, 71-77.

REUTHER G. & SALMINEN L. 1998 Influence of exogenouss growth regulators on endogenous hormones of *in vitro*-cultivated strawberries. pp. 11-20 in *Physiology and Control of Plant Propagation in vitro*. Proc. COST Workshop 822, Berlin 1996.

REVILLA M.A. & POWER J.B. 1988 Morphogenetic potential of long- term callus cultures of *Actinidia deliciosa*. J. Hort. Sci. 63, 541-545.

RHODES C.A., GREEN C.E. & PHILLIPS R.L. 1986 Factors affecting tissue culture initiation from maize tassels. Plant Sci. 46, 225-232.

RICE D. A. & SEDAT J.W. 2001 Histone methylation versus histone acetylation: new incites into epigenetic regulation. Curr. Opin. Cell Biol. 13, 263-273

RICE T.B. & CARLSON P.S. 1975 Genetic analysis and plant improvement. Annu. Rev. Plant Physiol. 26, 279-308.

RICE T.B., REID R.K. & GORDON P.N. 1978 Morphogenesis in field crops. pp. 262-277 *in* Hughes K.W., Henke R. & Constantin M. (eds.) 1978 *Propagation of higher plants through tissue culture.* Technical Inf. Center, U.S. Dept. Energy. Spingfield, Va. CONF-7804111.

ROBERTS L.W., GAHAN P.B. & ALONI R. 1988 *Vascular Differentiation and Plant Growth Regulators*. Springer-Verlag

ROBERTS D.R., FLINN B.S., WEBB D.T., WEBSTER F.W. & SUTTON B.C.S. 1989 The role of abscisic acid and indole-butyric acid in the differentiation and quality of somatic embryos of white spruce. Plant Physiol. 89, Suppl., 9.

ROBERTS. M. F. 1999 Phospholipases: generation of lipid-derived second messengers. *in* Sitaramayya A. (ed.) *Introduction to cellular signal transduction*. Birkhausser Verlag, Basel.

ROCA W.M., ESPINOZA N.O., ROCA M.R. & BRYAN J.E. 1978 A tissue culture method for the rapid propagation of potatoes. Am. Potato J. 55, 691-701.

ROEST S. 1977 Vegetative propagation *in vitro* and its significance for mutation breeding. Acta Hort. 78, 349-359.

ROEST S. & BOKELMANN G.S. 1975 Vegetative propagation of *Chrysanthemum morifolium* Ram. *in vitro*. Scientia Hort. 3, 317-330.

ROGLER C.E. & DAHMUS M.E. 1974 Gibberellic acid-induced phase change in *Hedera helix* as studied by deoxyribonucleic acid-ribonucleic acid hybridization. Plant Physiol. 54, 88-94.

ROSS. H. & ZOGLAUER. K. 1998 Phytohormone dynamics in relation to adventitous root formation of *Betula pendula* Roth. pp.37-45 in Reuther G. (ed.) *Physiology and Control of Plant Propagation In Vitro*. Proc. COST 822 Humboldt University Berlin 1996.

RUPERT E.A., SEO A., BROWNE W.C. & GIBSON P.B. 1976 Differentiation in callus and cell suspension cultures of *Trifolium* species and hybrids. Agronomy Abstracts, Am. Soc. Agron.

SACRISTAN M.D. & MELCHERS G. 1969 The caryological analysis of plants regenerated from tumorous and other callus cultures of tobacco. Mol. Gen. Genet. 105, 317-333.

SCARAMUZZI F. & DE GAETANO A. 1974 Studies on the rooting of shoot explants of *O. europea* cv. Ogliarola di monopoli grown *in vitro*. Ortoflorofrutticolt. Ital. 58, 419-436.

SCHRAUDOLF H. & REINERT J. 1959 Interaction of plant growth regulators in regeneration processes. Nature 184, 465-466.

SEABROOK J.E.A., CUMMING B.G. & DIONNE L.A. 1976 The *in vitro* induction of adventitious shoot and root apices on *Narcissus* (daffodil and narcissus) cultivar tissue. Can. J. Bot. 54, 814-819.

SEARS R.G. & DECKARD E.L. 1982 Tissue culture variability in wheat: callus induction and plant regeneration. Crop Sci. 22, 546-550.

RADOJEVIC L. 1979 Somatic embryos and plantlets from callus cultures of *Paulownia tomentosa* Steud. Z Pflanzenphysiol. 91, 57-62.

SHANNON J.C. & BATEY J.W. 1973 Inbred and hybrid effects on establishment of *in vitro* cultures of *Zea mays* L. endosperm. Crop Sci. 13, 491-493.

SHARP W.R. & EVANS D.A. 1982 Application of somatic embryogenesis to crop impovement. pp. 759-762 in Fujiwara A. (ed.) 1982 (q.v.)

SHARP W.R., LARSEN P.O., PADDOCK E.F. & RAGHAVAN V. (eds.) 1979 *Plant Cell and Tissue Culture*. Ohio State Univ. Press, Columbus

SHARP W.R., SONDAHL M.R., CALDAS L.S. & MARAFFA S.B. 1980 The physiology of *in vitro* asexual embryogenesis. Hort. Rev. 2, 268-310.

SHERIDAN W.F. 1974 A long term callus culture of *Lilium*: relative stability of the karyotype. J. Cell Biol. 63, 313a.

SHIMADA T. & MAKINO T. 1975 *In vitro* culture of wheat. III.Anther culture of the A genome aneuploids in common wheat. Theor. Appl. Genet. 46, 407-410.

SIM G.E., GOH C.J. & LOH C.S. 1989 Micropropagation of *Citrus mitis* Blanco - multiple bud formation from shoot and root explants in the presence of 6-benzylaminopurine. Plant Sci. 59, 203-210.

SIMON P.W. & PELOQUIN S.J. 1977 The influence of paternal species on the origin of callus in anther culture of *Solanum* hybrids. Theor. Appl. Genet. 50, 53-56.

SITARAMAYYA A. (ed.) 1999 *Introduction to Cellular Signal Transduction*. Birkhauser Verlag , Basel.

SMITH D.L. & KRIKORIAN A.D. 1988 Production of somatic embryos from carrot tissues in hormone-free medium. Plant Sci. 58, 103-110.

SMITH H. 2000 Phytochromes and light signal perception by plants – an emerging synthesis. Nature 407, 385-391.

SMITH S.M. & STREET H.E. 1974 The decline of embryogenic potential as callus and suspension cultures of carrot are serially subcultured. Ann. Bot. 38, 223-241.

SOMLEVA M.N., SCHMIDT E.D.L. & DE VRIES S.C. 2000 Embryogenic cells in *Dactylis glomerata* L. (Poaceae) explants identified by cell tracking and by SERK expression. Plant Cell Rep. 19, 718 – 726.

SOMMER H.E., BROWN C.L. & KORMANIK P.P. 1975 Differentiation of plantlets in Longleaf pine (*Pinus palustris*) tissue cultured *in vitro*. Bot. Gaz. 136, 196-200.

SONDAHL M.R. & SHARP W.R. 1977a High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. Z. Planzenphysiol. 81, 395-408.

SONDAHL M.R. & SHARP W.R. 1977b Growth and embryogenesis in leaf tissues of *Coffea*. Plant Physiol. 59, Suppl., 1.

SONDAHL M.R. & SHARP W.R. 1977c High frequency initiation of somatic embryos in cultured leaf explants of *Coffea arabica* L. In Vitro 13, 146.

SONDAHL M.R. & SHARP W.R. 1979 Research in *Coffea* spp. and applications of tissue culture methods. pp. 527-584 in Sharp *et al.,* (eds.) 1979 (q.v.).

SONDAHL M.R., SALISBURY J.L. & SHARP W.R. 1979a S.E.M. characterization of embryogenic tissue and globular embryos during high frequency somatic embryogenesis in coffee callus cells. Z. Pflanzenphysiol. 94, 185-188.

SONDAHL M.R., SPAHLINGER D.A. & SHARP W.R. 1979b A histological study of high frequency and low frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. Z. Pflanzenphysiol. 94, 101-108.

STAMP J.A. & MEREDITH C.P. 1988 Proliferative somatic embryogenesis from zygotic embryos of grapevine. J. Am. Soc. Hort. Sci. 113, 941-945.

STANDARDI A. & MICHELI M. 1988 Control of vitrification in proliferating shoots of M-26. Acta Hortic. 227, 425-427.

STIMART D.P., ASCHER P.D. & ZAGORSKI J.S. 1980 Plants from callus of interspecific hybrid of *Lilium* `Black Beauty'. HortScience 15, 313-315.

STREET H.E. & WITHERS L.A. 1974 The anatomy of embryogenesis in culture. pp. 71-100 *in* Street H.E. (ed.) 1974 *Plant Tissue and Plant Science.* Academic Press. London, New York, San Francisco.

STREET H.E. 1977 The anatomy and physiology of morphogenesis. Studies involving tissue and cell cultures. pp. 20-33 *in* Gautheret R.J. (ed.) 1977 *La Culture des Tissus des Végétaux. Résultat généraux et Réalisations practiques*. Masson, Paris.

STREET H.E. 1979 Embryogenesis and chemically induced organogenesis. pp. 123-153 in Sharp *et al.,* (eds.) 1979 (q.v.).

STUART D.A. & NELSEN J. 1988 Isolation and characterisation of alfalfa 7 S and 11 S seed storage protein. J. Plant Physiol. 132, 129-133.

SUDARSONO & GOLDY R.G. 1991 Growth regulator and axillary bud position effects on *in vitro* establishment of *Vitis rotundifolia*. HortScience 26, 304-307.

SUNG Z.R., SMITH J.M., CHOI J.H., KRAUSS M., BORKIRD C. & LIU L.-S. 1988 Gene expression in embryogenesis. HortScience 23, 513-515.

SWARTZ H.J. 1991 Post culture behaviour: genetic and epigenetic effects and related problems. pp. 95-121 in Debergh P.C.& Zimmerman R.H. (eds.) *Micropropagation. Technology and Application*. Kluwer Academic Publishers, Dordrecht, Boston, London. ISBN 0-7923-0818-2.

SZAKACS E., KOVACS G., JANOS P. & BARNABAS B. 1988 Substitution analysis of callus induction and plant regeneration from anther culture in wheat (*Triticum aestivum* L.). Plant Cell Rep. 7, 127-129.

TAEB A.G. & ALDERSON P.G. 1987 Micropropagation of tulip: optimising shoot production from floral stem explants. Acta Hortic. 212, 677-681.

TAIRA T., HASKINS F.A. & GORZ H.J. 1977 Callus and suspension cultures of *Melilotus alba* tissues and cells. Crop Sci. 17, 407-411.

TAKATORI F.H., MURASHIGE T. & STILLMAN J.I. 1968 Vegetative propagation of asparagus through tissue culture. HortScience 3, 20-22.

TAKAYAMA S. & MISAWA M. 1982 Factors affecting differentiation and growth *in vitro*, and a mass propagation scheme for *Begonia hiemalis*. Sci. Hortic. 16, 65-75.

TANAKA M., HASEGAWA A. & GOI M. 1975 Studies on the clonal propagation of monopodial orchids by tissue culture: 1. Formation of protocorm-like bodies (PLB) from leaf tissue in above sp. J. Jap. Soc. Hort. Sci. 44, 47-58.

TANIMOTO S. & HARADA U. 1980 Hormonal control of morphogenesis in leaf explants of *Perilla frutescens* Britton var. *crispa*, Decaisnef. *viridi-crispa* Makino. Ann. Bot. 45, 321-327.

TAUTORUS T.E., FOWKE L.C. & DUNSTAN D.I. 1991 Somatic embryogenesis in conifers. Can. J. Bot. 69, 1873-1899.

THAKUR S. & GANAPATHY P.S. 1978 Morphogenesis of organ differentiation in *Bacopa monnieri*: stem cultures. Ind. J. Exp. Biol. 16, 514-516.

THOMAS E., KING P.J. & POTRYKUS I. 1979 Improvement of crop plants *via* single cells *in vitro*. Z. Planzenzuchtg. 82, 1-30.

THOMAS T.L., CHUNG H-J. & NUNBERG. A.N. 1997 ABA signaling in plant development and growth. *In* Aducci P. (ed.) *Signal Transduction in Plants*. Birkhauser Verlag, Basel.

THORPE T. A. (ed.) 1978 *Frontiers of Plant Tissue Culture*. Int. Assoc. for Plant Tissue Culture, Univ. of Calgary, Calgary, Alberta, T2N 1N4 Canada.

THORPE T. A. 1980 Organogenesis *in vitro*: structural, physiological, and biochemical aspects. pp 71-111 in Vasil I.K. (ed.) 1980 *Perspectives in Plant Cell and Tissue Culture*. Int. Rev. Cytology Suppl. 11A. Academic Press.

TISSERAT B., ESAN E.B. & MURASHIGE T. 1979 Somatic embryogenesis in angiosperms. Hort. Rev. 1, 1-78.

TOIVONEN P.M.A. & KARTHA K.K. 1988 Regeneration of plantlets from *in vitro* cultured cotyledons of white spruce (*Picea glauca* Moench Voss). Plant Cell Rep. 7, 318-321.

TOMAR U.K. & GUPTA S.C. 1988 Somatic embryogenesis and organogenesis in callus cultures of a tree legume - *Albizia richardiana* King. Plant Cell Rep. 7, 70-73.

TORREY J.G. 1966 The initiation of organized development in plants. Adv. Morphogenesis 5 39-91.

TRAN THANH VAN M., DIEN N.T. & CHLYAH A. 1974 Regulation of organogenesis in small explants of superficial tissue of *Nicotiana tabacum* L. Planta 119, 149-159.

TRAYNOR P.L. & FLASHMAN S.M. 1981 Hormone-induced caulogenesis in long-term tobacco cell lines and its effect on nuclear DNA content. Theor. Appl. Genet. 60, 31-36.

TREWAVAS A.J., RODRIGUES C., RATO C. & MALHO R. 2002 Cyclic nucleotides: the current dilemma! Curr. Opin. Plant Biol. 5 (5), 425-429.

TRIGIANO R.N., GRAY D.J., CONGER B.V. & McDANIEL J.K. 1989 Origin of direct somatic embryos from cultured leaf segments of *Dactylis glomerata*. Bot. Gaz. 150, 72-77.

URAGAMI U., SAKAI A., NAGAI M. & TAKAHASHI T. 1990 Cryopreservation by vitrification of cultured cells and somatic embryos from mesophyll tissue of asparagus. Acta Hortic. 271, 109-116.

VAN DEN ENDE G., CROES A.F., KEMP A. & BARENDSE G.W.M. 1984a Development of flower buds in thin-layer cultures of floral stalk tissue from tobacco: role of hormones in different stages. Physiol. Plant. 61, 114-118.

VAN DEN ENDE, G., CROES, A.F, KEMP, A., BARENDSE, G.W.M. & KROH, M. 1984b Floral morphogenesis in thin-layer tissue cultures of *Nicotiana tabacum*. Physiol. Plant 62, 83-88

VASIL V. & VASIL I.K. 1981 Somatic embryogenesis and plant regeneration from tissue cultures of *Pennisetum americanum* and *P. americanum* x *P. purpureum* hybrid. Am. J. Bot. 68, 864-872.

VASIL V. & VASIL I.K. 1982 The ontogeny of somatic embryos of *Pennisetum americanum* (L.) K. Schum. I. In cultured immature embryos. Bot. Gaz. 143, 454-465.

VENIS M.A. & NAPIER R.M. 1997 Auxin perception and signal transduction. *in* Aducci P. (ed.) *Signal Transduction in Plants*. Birkhauser Verlag, Basel.

VENKETESWARAN S. & MAHLBERG P.G. 1962 Proliferation of albino and pigmented genetical strains of *Nicotiana* tissue culture. Physiol. Plant. 15, 639-648.

VERMA D.C. & DURZAN D.J. 1980 Somatic embryogenesis in cell suspension cultures of wild carrot in a `tissue culture cluster dish' (Costar). Plant Physiol. 65, Suppl., 90.

VIEITEZ F.J., SAN-JOSE C., BALLESTER A. & VIEITEZ A.M. 1990 Somatic embryogenesis in cultured immature zygotic embryos in chestnut. J. Plant Physiol. 136, 253-256.

VILLALOBOS V.M., YEUNG E.C. & THORPE T.A. 1984 Origins of adventitious shoots in excised radiata pine cotyledon *in vitro*. Can J. Bot. 63, 2172-2176.

VOET D., VOET J.G. & PRATT C.W. 1999 *Fundamentals of Biochemistry*. John Wiley & Sons, New York.

VON ARNOLD S. & ERIKSSON T. 1978 Induction of adventitious buds on embryos of Norway spruce grown *in vitro*. Physiol. Plant. 44, 283-287.

WAN Y., SORENSEN E.L. & LIANG G.H. 1988 Genetic control of *in vitro* regeneration in alfalfa (*Medicago sativa* L.). Euphytica 39, 3-9.

WANG L., WYNDAELE R. & GAHAN P.B. 2002 Exogenously applied plant bioregulator induction of vessels in cotyledons of *Lycopersicon esculentum*. Plant Cell Tissue Organ Cult. 70, 213-221.

WANG W.C., TRONCHET M., LARROQUE N., DORION N. & ALBOUY J. 1988 Production of virus-free *Dahlia* by meristem culture and virus detection through cDNA probes and ELISA. Acta Hortic. 234, 421-428.

WEBB J.K., BANTHORPE D.V. & WATSON D.G. 1984 Monoterpene synthesis in shoots regenerated from callus cultures. Phytochemistry 23, 903-904.

WEBB K.J., FAY M.F. & DALE P.J. 1987 An investigation of morphogenesis within the genus *Trifolium*. Plant Cell Tissue Organ Cult. 11, 37-46.

WEHNER T.C. & LOCY R.D. 1981 *In vitro* adventitious shoot and root formation of cultivars and lines of *Cucumis sativus* L. HortScience 16, 759-760.

WELANDER M. 1988 Plant regeneration from leaf and stem segments of shoots raised *in vitro* from mature apple trees. J. Plant Physiol. 132, 738-744.

WERNICKE W., POTRYKUS I. & THOMAS E. 1982 Morphogenesis from cultured leaf tissue of *Sorghum bicolor* - The morphogenic pathways. Protoplasma 111, 53-62.

WICKHAM K., RODREGUEZ E. & ARDITTI J. 1980 Comparative phytochemistry of *Parthenium hysterophorus* L. (Compositae) tissue cultures. Bot. Gaz. 141, 435-439.

WILLIAMS E.G. & MAHESWARAN G. 1986 Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. Ann. Bot. 57, 443-462.

WINTON L.W. 1968 The rooting of liquid-grown aspen cultures. Am. J. Bot. 55, 159-167.

WOLFFE A.P. & MATZKE M.A. 1999 Epigenetics: Regulation through repression. Science 286, 481-486.

WOLFSBERG T.C., MCENTYRE J. & SCHULER G. 2001 Guide to the draft human genome. Nature 409, 814-816.

WOODWARD A.W. & BARTEL B 2005 A receptor for auxin. The Plant Cell 17, 2425-2429.

WYNDAELE R., CHRISTIANSON J., HORSEELE R., RÜDELSHEIM P. & VAN ONCKELEN H. 1988 Functional correlation between endogenous phytohormone levels and hormone autotrophy of transformed and habituated soybean cell lines. Plant Cell Physiol. 29, 1095-1101.

YAMAZAKI A. 1999 Cyclic nucleotides: synthesis by adenyl and guanyl cyclases. *in* Sitaramayya A. (ed.) *Introduction to Cellular Signal Transduction*. Birkhausser Verlag, Basel.

YANAGAWA T. & SAKANISHI Y. 1980 Studies on the regeneration of excised bulb tissues of various tunicated bulbous ornamentals. I. Regenerative capacity of the segments from different parts of bulb scales. J. Jap. Soc. Hort. Sci. 48, 405-502.

ZEE S.-Y., & WU S.C. 1979 Embryogenesis in the petiole explants of Chinese celery. Z. Pflanzenphysiol. 93, 325-335.

ZILBERMAN D., CAO, X., & JACOBSON S. E. 2003 ARGONAUTE4 control of locus specific siRNA accumulation and DNA and histone methylation. Science 299, 716-719

ZIMMERMAN T.W., ROGERS S.M. & COBB B.G. 1988 Physiological differences between normal and vitreous petunias grown *in vitro*. HortScience 23, 780.

ZIV M., HALEVY H. & SHILO R. 1970 Organs and plantlets regeneration of gladiolus through tissue culture. Ann. Bot. 34, 671-676.

Chapter 11
Stock Plant Physiological Factors Affecting Growth and Morphogenesis

1. INTRODUCTION

Most *in vitro* studies focus on manipulating the medium. By varying the nutrient salts (see Chapter 3), gelling agents (Chapters 3 and 4), and especially plant growth regulators (Chapters 5-7), it has been possible to grow callus, stimulate axillary shoot proliferation and rooting, and induce shoot organogenesis (Chapter 10) and somatic embryogenesis (see Chapters 8 and 9). However, *in vitro* growth and morphogenesis are also governed by properties of the explant. It is well known that different genotypes will often not respond in the same way when cultured on the same medium. This has required countless empirical studies on medium optimization for different species or even cultivars of the same species. The effects of the plant genotype on *in vitro* growth and morphogenesis were described in the previous chapter.

Within a plant during its growth and development, different parts of the genetic code are expressed differentially in different cells (see Chapter 8). This results in distinct patterns of plant growth and morphogenesis *in vitro* and *extra vitrum*. Such phenotypic expression is the result of an interaction between the genotype and the environment. We choose the genotype by selecting the species and/or cultivar. We manipulate the gene expression by changing the environment to which the explant or the stock plant is exposed. Other factors also influence gene expression, including the tissue or organ selected for explant excision, season or growing conditions of the stock plant and the ontogeny of the stock plant.

When different genes are "turned on" or "turned off" in a cell or even a few cells, it can have profound influences on plant growth and development and phase change (Hackett and Murray, 1997; Poethig, 2003). This differential expression is referred to as epigenetic variation. Control of epigenetic variation is the focus of plant propagation (Hartmann *et al.,* 2002) and tissue culture.

2. EPIGENETIC EXPRESSION AND CELL DETERMINATION

Plants cells have an amazing ability to respond to a signal, which almost invariably is a plant growth substance. When the cells can respond, they can change from a previously determined developmental pathway. For example, a leaf cell can change so that it will begin to divide and a root or shoot will form.

In tissue culture and plant propagation, the ratio of cytokinin : auxin is important (Chapter 6). When cytokinin is in excess, adventitious shoots will form, when auxin is in excess, adventitious roots or somatic embryos will form, and when there are moderate to high levels of both, callus will develop. This is the normal wound healing process in plants. Many plant species have the remarkable ability to replace wounded or lost organs. Following wounding, the endogenous hormone levels change and callus formation and organogenesis are often the result. Similarly, with some species, because of high auxin levels associated with sexual fertilization in the seed, adjacent somatic cells change and produce embryos through the process of apomixis. In plant propagation and plant tissue culture, we exploit this healing process and the apomictic ability of cells.

Different cells within a tissue will not all respond the same way to a change in the cytokinin : auxin ratio. Understanding what distinguishes certain cells from others in their ability to respond to the plant growth substance is important. As our knowledge of the molecular biology of cells increases we may understand why there are these cellular differences. With such knowledge, we may gain the ability to regenerate recalcitrant genotypes, such as adult forms of many plants.

Christianson (1987) and Christianson and Warnick (1983, 1988) stated that there are competent cells in a plant or explant (see Chapter 10). These are the cells that are capable of recognizing the plant growth regulator signal and changing, or becoming determined. When the plant growth regulator signal is removed, the determined cells will continue to respond. They will dedifferentiate (become meristematic again), and their daughter cells will differentiate into the new shoot, root, or somatic embryo.

Not all cells in a tissue are competent. Exactly what makes one cell competent to perceive a plant

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growth regulator signal and its adjacent cell not to be competent is not well understood. It is known that applied plant growth regulators elicit specific mRNA molecules (Christianson and Warnick, 1988). This is evidence of specific genes being expressed in response to the exogenous plant growth substances. However, this specific gene expression is more clearly related to changes in determined cells than to what makes cells competent.

A problem with trying to understand what makes a cell competent is that it is difficult to ascertain that a specific cell is competent until after it responds to the plant growth substances and becomes determined. Once determined, the cell changes and its gene expression can be studied. However, it is difficult to know which cell is competent and which one is not before the cell becomes determined.

It is likely that epigenetic expression is the reason that a cell is competent. Perhaps there is a gene or genes expressed for the receptor molecule of the elicitor plant growth regulator, which would allow that competent cell to recognize the plant growth substance. However, there are other possibilities that could lead to competence, such as a expression of an important gene in the signal transduction pathway in a competent cell.

The predetermination of cells and tissues can often be changed in culture by cell differentiation and cell division, leading to the direct formation of organogenic or embryogenic meristems or callus. But if the tissue is not competent to undergo these changes, or the growth regulators in the medium are not suitable, previously predicated developmental pathways may not be interrupted. Some aspects of determination may nevertheless survive in newly-established cultures and can occasionally persist for many callus transfers.

2.1. PHASES OF GROWTH

The sequence of stages through which a higher plant has passed in its development from a fertilized egg to an adult organism, is known as its ontogeny or ontogenesis.

In the juvenile phase, a young seedling plant displays one or more distinctive characteristics of both a morphological and physiological nature. These distinguish a juvenile plant from an adult. The juvenile phenotype gradually disappears during subsequent growth, and under natural conditions it is replaced by the adult (mature) phase in those parts of the plant in which maturation has occurred. Maturation occurs when the apical meristems become capable of changing from vegetative to flowering meristems under "normal" inductive conditions. In some plants, a transitional phase of development can be distinguished between the juvenile and adult phases during which the potential to flower gradually increases.

When a plant is induced to flower, the shoot apical meristem changes from forming vegetative structures to forming reproductive organs (Hackett and Murray, 1997). Typically, it is an external environmental stimulus, such as day length or chilling that will trigger this change. Under such a normal environmental stimulus the apical meristem of a juvenile plant is not capable of perceiving or responding to the signal and remains vegetative. Under certain circumstances, juvenile plants can be induced to initiate flowers but will revert to the non-flowering juvenile state for several years (Hackett, 1987). However, it is often difficult to devise methods to promote flowering that will not also advance maturation (Hackett, 1985). In many conifers, flowering can be induced on juvenile shoots by gibberellins or auxin/gibberellin combinations (Pharis and King, 1985; Pharis and Kuo, 1977; Pharis *et al.,* 1976, 1980; Ross *et al.,* 1981).

In general, the duration of the juvenile phase is proportional to the potential ultimate size of the plant, being shortest in annual herbaceous species and progressively longer and more noticeable in perennial and woody plants. In trees it may sometimes persist for many years.

Phase change from juvenile to adult is an epigenetic change, in that there are phenotypical changes that are the result of changing gene expression, not mutation. When an adult plant is regenerated from an embryo (either zygotic or somatic), juvenile traits are again expressed. Gene expression for dihydroflavonol reductase (*DFR*) was studied in adult and juvenile leaf lamina tissue of English Ivy (Hackett and Murray, 1997). *DFR* activity was detected in juvenile leaves treated with sucrose and light, but not in mature phase discs. It was determined that the lack of DFR activity was because there was no accumulation of *DFR* mRNA because of a lack of transcription of the *DFR* gene in mature phase discs. Lack of expression of this gene was also reported for adult phase stem tissue. This gene was expressed in juvenile leaves and stems. The specific reason why the *DFR* locus was not transcribed was unknown. In *Arabidopsis*, both light and temperature independently regulate the floral promoter (*FT*) gene (Poethig, 2003). However, there

are other genes that also regulate temperature sensitivity of flowering time. These genes (*FCA* and *FVE*) act upstream from the *FT* gene and seem to be involved in phase change.

2.2. CHARACTERISTICS OF JUVENILE PLANTS

There are numerous substantive phenotypic traits associated with juvenility, but they vary considerably among species. Commonly, the leaves on young plants are of a different shape than those on mature parts and may be simple rather than compound (or occasionally the reverse); juvenile leaves may also have a special type of cuticle and be arranged with a distinct phyllotaxy. Compared to their adult counterparts, young plants may have a modified resistance to pests and diseases. Juvenility in woody plants is often manifested by prolonged vigorous shoot growth. With *Citrus* and *Gleditsia triacanthos*, juvenile forms are thorny, whereas adult forms lack the thorniness and with some tree species, such as those of *Quercus* and *Fagus,* juvenile forms and older, more juvenile parts of the trees, hold their senesced leaves throughout the winter.

2.2.1. Vegetative propagation

To the plant propagator, the most important attribute of juvenile shoots is their ability to provide cuttings that readily form adventitious roots or explants that respond and grow well *in vitro*. Cuttings taken from adult shoots of plants can be rooted, but the frequency of success is often low, especially with woody plants. Likewise, researchers have had great challenges when attempting to micropropagate adult forms of many woody species. The change from the juvenile to adult phase is the most serious constraint to rooting in shrubs and trees (Howard, 1990). Most of the difficulty experienced in rooting mature shoots seems to be caused by their altered physiology, but can also be related to greater contamination with microorganisms and viruses.

Hedera helix has been used widely to study juvenility because it has a distinctively different morphology between the juvenile and adult phases. Juvenile plants have a different growth habit, leaf shape, and an enhanced ability to form adventitious roots. When petioles from the juvenile form were excised and treated with auxin *in vitro*, cortical parenchyma cells adjacent to the vascular bundles divided and formed root primordia (Geneve *et al.,* 1988). However, when petioles from adult leaves were treated in a similar manner, callus formed and some callus cells divided to form root primordia. The juvenile form had pre-existing competent cells that were able to respond to auxin and become determined to form roots. However, the adult form appeared to lack cells with pre-existing competence to form roots, but competence was acquired by some callus cells once they had been initiated.

Explants taken from mature shoots are frequently more liable than juvenile material to suffer necrosis, especially when surface disinfested and placed in culture (Hanus and Rohr, 1987). For example, shoot tip explant death can occur within a few hours for adult *Juglans nigra* whereas healthy growth was evident on seedling explants when both sources were compared in different vessels containing the same medium (Preece and Van Sambeek unpublished). It was only by changing the medium and culture conditions that adult *J. nigra* shoot cultures have been maintained for years (Pearson and Preece unpublished). However, adult origin *J. nigra* microshoots still cannot be rooted.

For tissue culture, juvenile explants are usually more readily established *in vitro* and grow and proliferate at a more rapid rate than adult material. This is particularly true with tree species where micropropagation of adult material is often difficult.

2.2.2. Plant propagation dilemma

It is well known that it is easier to propagate vegetatively, juvenile forms of plants than adult forms of plants. When breeding and selecting new, superior plants for clonal propagation, it is usually necessary to wait until the plant reaches maturity. This allows for evaluation of important features, such as ultimate form and size, flowering and fruiting characteristics, autumnal coloration, and other traits. At the point that the mature phenotype is known, the plant is an adult and often becomes difficult to propagate clonally. Libby and Hood (1976) showed that juvenility can be maintained by hedging radiata pine. They rooted cuttings of many juvenile selections and by allowing some individuals to grow to maturity for evaluation, other, hedged members of the same clone could be maintained as juvenile plants for propagation. This technique also has potential for micropropagation.

2.3. THE PROGRESSION OF PHASIC DEVELOPMENT

The juvenile or adult phase is expressed by many cells within a plant, but is particularly evident in the apical meristems from which new organs are initiated. The meristems of juvenile shoots are usually smaller than those on their mature

counterparts and are composed of cells with small nuclei. Meristems are thus determined to give rise to juvenile or adult organs or tissues in the same way that they can be programmed to give rise to shoots, roots or differentiated cells.

Both juvenile and adult phases of growth result from persistent and stable gene expression in the shoot meristems. As cells divide, the expression is transmitted to daughter cells, and continues when shoots are propagated from cuttings or grafting. It may then be gradually changed by environmental conditions or be retained for several years. The change of an apical meristem from a juvenile condition to one with the form and function of the adult phase (and vice versa), generally takes place progressively. Some features of the plant phenotype may change at a different pace than others. For example, although seedling trees of Douglas fir have not been found to flower until they are 20 years-old, 100% of cuttings from a 9 year-old tree could be rooted, but only 5% of those from trees aged 14-24 years (Black, 1972). For some characters, particularly the ability to flower, the switch from one phase to the other is more abrupt and the phenotypic characteristics of each phase quite distinct. In many *Eucalyptus* species, leaf shapes are distinct, either juvenile, intermediate, or adult (Durand-Cresswell *et al.,* 1982).

When a plant is juvenile, it puts on juvenile growth. This becomes the oldest part of a plant as it ages (Fig. 11.1). These lower and older portions of the plant retain their juvenile characteristics. On a tree, this includes the trunk and the portion of the lower branches near the trunk. Some temperate tree species, such as *Quercus* (oak), *Fagus* (Beech), and some *Acer* species (maple) retain their leaves throughout the winter as a juvenile characteristic. Viewing mature specimens of these species during the winter and focusing on the portion still holding its leaves, allows visualization of the shape of the tree when it was young and juvenile. This older, but more juvenile portion of the plant is called the 'cone of juvenility.'

It is not until a plant is sufficiently old and large that it goes through phase change and develops adult characteristics. These characteristics will manifest themselves on the newest growth. Therefore, the outside and higher portions of a mature tree are the most adult. There can be a transition region between the cone of juvenility and the adult portion of the plant that is represented by the new growth (Fig. 11.1).

Fishel *et al.,* (2003) dissected the main stems of six 10-15 year-old, 7-9 m tall *Quercus rubra* (northern red oak) trees by cutting them at the base and then into 40 cm long sections, identified as to their original location on the tree. These stem segments were placed under intermittent mist and softwood shoots were forced, harvested and rooted. Both softwood shoot production and rootability of the shoots was greatest from the lowest segments and declined with increasing section height. This is consistent with a gradual gradient of maturation from the base (most juvenile) to the top (most adult). However, shoot production was consistent throughout *Quercus bicolor* (swamp white oak) stem segments, indicating species differences.

The juvenile form appears in new growth from seedlings and seems to be associated with the proximity of shoot apical meristems to the roots of the plant, and the presence of a normal unrestricted root system. As the physical distance between the shoot tips and the roots increases with growth, the juvenile condition of shoot meristems disappears. *Nicotiana sylvestris* plants do not flower if, each time the plants begin to initiate flowers, their shoot tips are removed and re-rooted (Dennin and McDaniel, 1985). Micropropagated conifers develop adult features early in their development (Mohammed and Vidaver, 1988). This may be because the plants have poorly developed root systems.

2.4. REJUVENATION

Once the adult phase has been reached in a plant, it is more or less stable (Hackett, 1987). This is problematic in both clonal macro- and micropropagation since adult propagules respond poorly.

In fruit trees, such as apple, repeated pruning (hedging) or severe annual pruning back to the ground level in winter can be successful in inducing the formation of rejuvenated shoots with an improved rooting ability. It is a drastic step to cut down a mature specimen plant to be able to produce juvenile shoots from the stump for propagation purposes. When non-flowering shoots with juvenile characteristics are formed on mature woody plants, they are termed juvenile reversion shoots.

Although shoots arising from latent buds (epicormic axillary or adventitious buds buried under the bark on woody species) on mature plants usually have a juvenile morphology, they generally give rise to plants that flower in a shorter time than seed-derived plants and so may not be completely juvenile.

The features of juvenility found on epicormic and basal shoots can be short-lived and liable to disappear quickly as the shoots elongate. The roots from which suckers arise seem to retain a juvenile state for a long period.

New shoots with some juvenile characteristics often appear from swellings or outgrowths on the trunks or at the bases of a mature trees. Such swellings are known as spheroblasts (nodular growths), or lignotubers (swellings at the base of the stem which arise on the lower nodes of seedlings) (James, 1984; Fig. 11.1)

These structures have been used by propagators to produce shoots for vegetative propagation purposes for over one hundred years (Preece, 2003). These shoots do not always arise adventitiously, and may sometimes grow from buds formed during the early life of the tree and have remained suppressed and dormant. They can be induced to form on the lower part of trees by partial girdling, coppicing, or other damage to the plant.

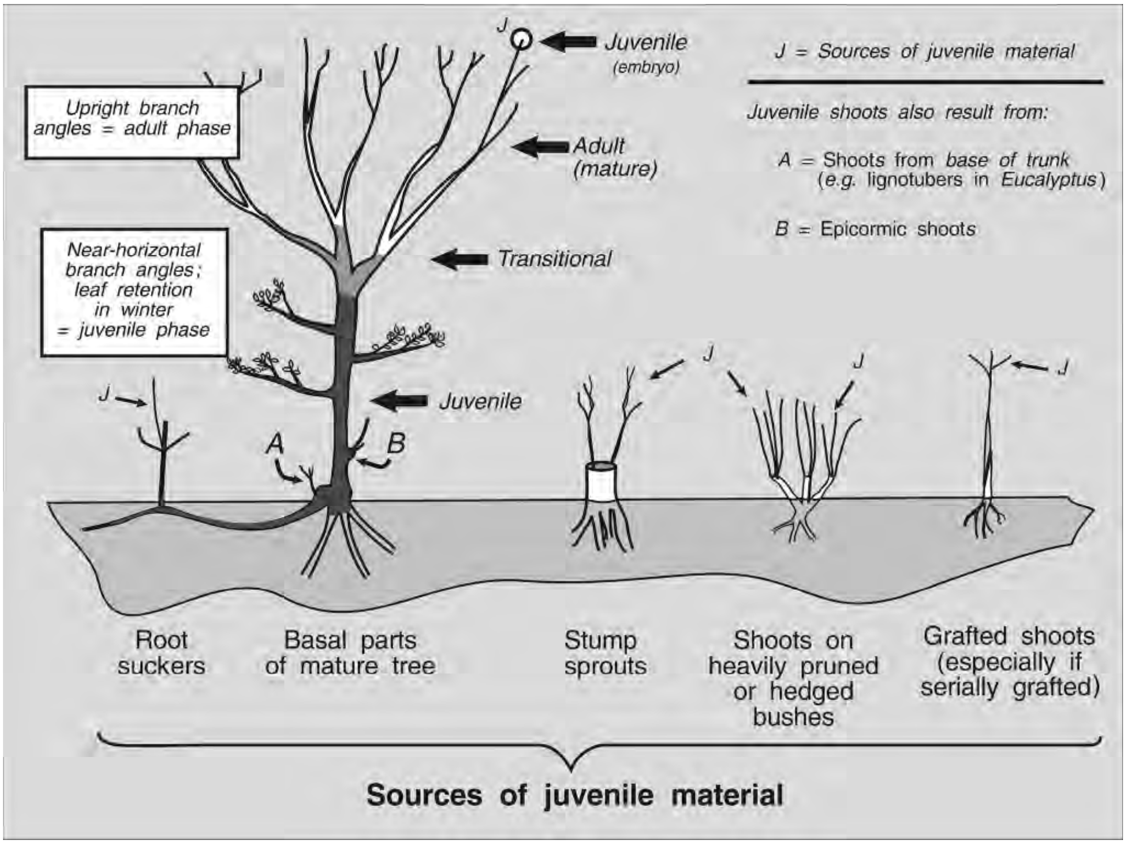

Fig. 11.1 Some typical juvenility patterns in a deciduous dicotyledonous tree, showing where juvenile shoots may be obtained.

2.5. NATURAL REVERSION

It has been proposed that flower formation is the stage at which juvenility is normally re-initiated (Meyer, 1984). A reversion to a juvenile state certainly seems to occur within the maternal tissues of the floral organs from which zygotic embryos are derived; it is fully apparent in zygotic embryos, but is not related to sexual fertilization, because apomictic embryos also give rise to juvenile seedlings [for example, plants derived from the nucellar embryos of *Citrus* are juvenile (Swingle, 1932 in Brink, 1962)]. An indication of the juvenile status of inflorescences, flowers and embryos, is that they have often regained an ability to form adventitious shoots and somatic embryos *in vitro*. Therefore floral organs and immature fruit are often selected as explants because they respond well *in vitro*, for example Jorgensen (1989) produced somatic embryos from callus from filaments collected from *Aesculus hippocastanum*

(horsechestnut) trees up to 100 years old. Plantlets derived from these sources have juvenile characteristics.

There are exceptions to this. When rhododendron flower parts were placed *in vitro*, adventitious shoots formed (Shevade and Preece, 1993). Additionally, adventitious floral structures formed, which is a sign of maturation. Although adventitious shoots also formed from leaves (Preece and Imel; 1991, Preece *et al.,* 1993), no adventitious floral structures formed from leaf explants. Therefore, the *in vitro* process does not always alter the epigenetic expression of maturation.

2.6. INDUCTION OF JUVENILITY

2.6.1. Forcing epicormic shoots

Softwood shoots can be forced to grow from large stem segments placed in controlled environments (George and Preece, 2004, Henry and Preece, 1997a, b; Ledbetter and Preece, 2003, Preece and Read, 2003, Preece *et al.,* 2002, Van Sambeek *et al.,* 1997a,b; Van Sambeek and Preece, 1999; Vieitez *et al.,* 1994). Forcing is accomplished by cutting branch or main stem segments from woody plants 25 cm to $\geq$2 m long, laying horizontally in a suitable medium such as perlite for mist or vermiculite in the laboratory or greenhouse, then allowing latent, axillary, or adventitious buds to elongate. If the stem segments are excised from within the cone of juvenility, it is expected that the new, softwood shoots will have juvenile characteristics. This varies with species (Fishel *et al.,* 2003). The best forcing environment tested has been under intermittent mist (Van Sambeek and Preece, 1999). However, if explants are to be excised for *in vitro* purposes, intermittent mist is not acceptable because it results in high microbial contamination. Rather, for *in vitro* use, irrigation must be such that water does not contact the new shoots. Then it is possible to establish explants aseptically (Van Sambeek *et al.,* 1997a, 1997b; Vieitez *et al.,* 1994).

Shoot forcing from large stem segments can allow for the excision of juvenile shoots from adult plants. This method does much less damage to a plant than cutting it down to the soil line to force new suckers with juvenile characteristics. Using large stem segments from within the cone of juvenility may have more potential than forcing softwood shoots from the more adult outside portions of a plant.

Another advantage of forcing shoots for propagation is that they can be rooted better for at least some species than cuttings from field-grown plants. For example, Fishel *et al.,* (2003) reported that forced cuttings from lower stem segments of northern red oak rooted better than cuttings from field-grown stump sprouts or branch tip cuttings 1-4 m above the ground. Ledbetter and Preece (2003) reported that softwood shoots forced from large stem segments under intermittent mist produced more roots than softwood shoots from outdoor-grown *Hydrangea quercifolia* (oakleaf hydrangea). In both cases it may be that shoots that are produced under intermittent mist are more adapted to the misting environment than outdoor-grown cuttings.

2.6.2. Re-rooting and grafting

A partial juvenility or rejuvenation can sometimes be restored on mature shoots by rooting the apex as a cutting (Lee and Rao, 1986), especially if the rooting process is repeated several times (Dennin and McDaniel, 1985), i.e. a cutting is taken and after a short period of growth on its own roots, its apex is removed and re-rooted.

Shoots (and particularly isolated shoot apices) grafted onto juvenile rootstocks can also regain a juvenile status (Debergh and Maene, 1985), but again the process may have to be repeated several times ('serial grafting') to reinforce juvenile traits (Bonga and Von Aderkas, 1992, Fig. 11.2). The rejuvenation of adult shoots by grafting is stimulated by leaves on the juvenile rootstock, but inhibited by leaves on the adult scion (Hackett, 1976). Successful rejuvenation also depends on there being little mature wood on the scion (Stoutemeyer and Britt, 1961). In practice, therefore, single or serial micrografting is frequently used for rejuvenation using small shoot tips as scions (Franclet, 1987; Monteuuis *et al.,* 1987; Pliego-Alfaro and Murashige 1987). Giovannelli and Giannini (1999) compared the performance of explants collected from adult *Castanea sativa* (chestnut) plants and from the fourth serial graft. Explants from the adult trees had poor shoot proliferation and 50% showed apical necrosis and death, whereas rejuvenated explants from the serial grafts proliferated well and the microshoots rooted better than microshoots from the adult explants.

2.7. ETIOLATION

The development of plants or plant parts in the absence of light is known as etiolation. This treatment may also improve the facility with which explants from adult material can be cultured *in vitro*. It has, for example, been found to be an effective treatment for preparing mature sweet chestnut shoots for culture (Ballester *et al.,* 1989). In late May (early

summer), newly-grown, 10-15 cm long sweet chestnut shoots were stripped of leaves and (while still on the tree), wrapped in foil for 4 months. When shoot explants were taken from this material, not only was a high proportion established *in vitro* (79% vs. 22% in the controls), but there was also a rapid rate of shoot proliferation. From these results, it was proposed that etiolation brings about physiological rejuvenation of adult shoots, because it has no beneficial effect on seedling material (Hansen and Lazarte, 1984; Bennett and Davies, 1986). It is most effective when applied to the new young shoots that arise from severely pruned plants.

2.8. HIGH TEMPERATURES

Above-normal temperatures have been found to promote the growth of juvenile shoots from adult plant material (Fisher, 1954; Hudson and Williams, 1955). Reversal to the juvenile phase was more pronounced in *Hedera canariensis* if grafts of adult scions onto juvenile rootstocks were grown at 27°C, rather than at lower temperatures (Stoutemeyer and Britt, 1961). Providing bottom heat at 15°C above the ambient temperature, induced the formation of juvenile basal shoots in potted plants of *Hamamelis japonica*, *Magnolia soulangeana* and *M. stellata* (Debergh and Maene, 1985). The shoots were sufficiently re-juvenated to furnish suitable explants for tissue cultures.

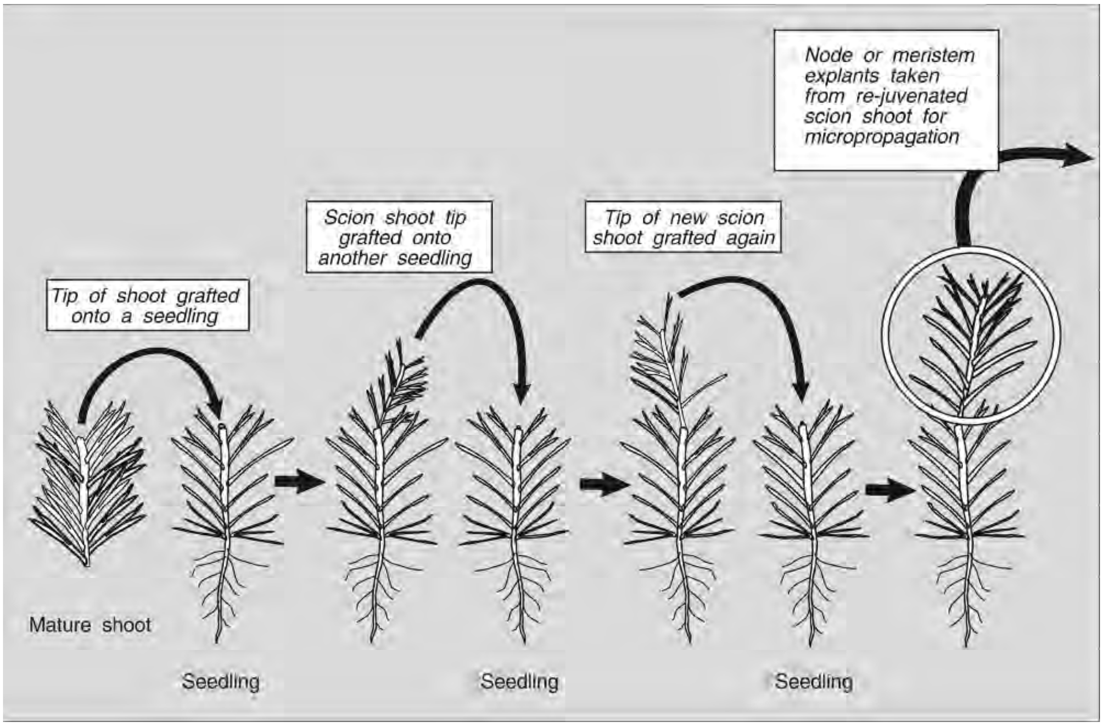

Fig. 11.2 Cascade grafting to induce rejuvenation. The drawing shows the use of the technique in a gymnosperm. The same method can be used for broad-leafed plants.

2.9. GROWTH REGULATOR TREATMENTS

Spraying (Abo El-Nil, 1982) or injecting adult shoots of some plants with cytokinins can induce a reversion to a semi-juvenile state. This treatment may also increase the number of axillary shoots on the plant from which explants can be taken. To obtain shoot material that is sufficiently rejuvenated for tissue culture, spraying with cytokinin is sometimes preceded by one or more of the other treatments mentioned above, such as taking cuttings, hedging, or grafting (Clark, 1982; Pierik, 1990a,b). Perez-Parron, *et al.,* 1994, had higher bud sprouting from adult *Fraxinus angustifolia* shoots collected in the winter when the forcing solution contained BA, compared to NAA or GA_3. However, cytokinin applications are not always effective for stimulating rejuvenation (Hackett, 1985).

A juvenile phase of growth can sometimes be induced on adult forms of *Hedera helix* plants by spraying them with GA_1, GA_3, or GA $_{4+7}$ (Hackett, 1985). However, treating mother plants with gibberellin can result in poor explant establishment from some species [e.g. meristem tips of *Pistacia vera* (Barghchi, 1986a)]: it may also result in the failure of explants or cuttings to form adventitious roots and so should be used with caution. Spraying woody stock plants with a mixture of a cytokinin and a gibberellin can also be effective in inducing vigorous (juvenile) new growth.

2.10. THE EFFECT OF PHASE OF GROWTH ON TISSUE CULTURES

In many woody plants, it is much easier to establish juvenile than adult explants *in vitro*. There are far more examples of successful micropropagation of juvenile forms of woody species than adult forms. Adult explants often produce more phenolic substances that blacken the medium than juvenile explants. For example, *Juglans nigra* adult shoot and nodal explants produce and release phenolic substances that oxidize, darken the medium, and if measures are not taken, will be autotoxic and kill the explants (Van Sambeek *et al.,* 1997a). In this study, 16% of juvenile explants were lost because of explant decline and exudation during establishment, whereas 31% of adult explants were lost for the same reason. One of the best methods to overcome the problem with exudation is to transfer to fresh medium every day or two during the first two weeks *in vitro*. With *J. nigra*, even when established, cultures must be transferred to fresh medium every two weeks. If this is done every three weeks, the cultures will decline and if monthly, shoot cultures will not survive for long. It was necessary to trim the bases of *J. regia* shoot explants and transfer them at 2 day intervals during the first 3 months of culture (McGranahan *et al.,* 1988a). After this time, cultures were more easily managed and only required to be transferred at 7 day intervals.

Besides affecting establishment, the relative maturity of the explant nearly always has major effects on the growth and morphogenesis of plant tissues and organs in culture. Zygotic embryos or pieces taken from germinated seedlings are therefore reliable explants for embryogenesis or shoot organogenesis. Adult-source explants generally are much less responsive.

There are numerous examples in the literature to show that explants derived from the juvenile phase are most responsive in culture. For example, the rate of survival and the subsequent growth rate of juvenile blueberry (*Vaccinium ashei*) explants is much higher than those of adult explants (Smagula and Lyrene, 1984). The most rapid initial shoot proliferation of *Prunus avium* shoot cultures was from explants derived from root suckers of a mature tree (Pevalek-Kozlina and Jelaska, 1987). In cultures established from 1 year-old and 5 year-old trees, the rate was lower, and explants from mature trees could not be established. McCown (1989) reported that with *Betula* spp., it is possible to obtain similar results with juvenile and adult cultures if time is allowed to stabilize the adult cultures. It took over three years *in vitro* where the tissue was subcultured every month to get the adult cultures to multiply well. It is possible the mature birch was rejuvenated by prolonged culture *in vitro*.

2.11. REJUVENATION

In the genus *Castanea*, Chevre and Salesses (1987) found that shoot tips from seedlings could be placed *in vitro* and propagated by shoot culture, but adult material (for example of the hybrid *C. crenata x C. sativa*) required rejuvenation first in layer beds. Babu *et al.,* (2000) excised nodal explants from root suckers from a mature, 7 year-old *Murraya koenigii* (curry leaf tree) and multiple shoots developed. Onay (2000) collected 3-4 cm long terminal lignified stem sections from 30 year-old *Pistacia vera* trees. Stems had their bases soaked in 44 µM BA for 24 hours prior to planting in a sand/soil mix and placed in the greenhouse. After 3 weeks, 10% of the stems had actively growing shoots that were excised and placed *in vitro*. These explants produced multiple shoots after 30 days. Only single node cuttings, and not axillary and terminal buds from 2 year-old *Feijoa sellowiana* seedlings would produce shoots in culture (Bhojwani *et al.,* 1987). The response of single node cuttings declined with age, and only 12% of those from a 3 year-old plant sprouted *in vitro*. However, nodal explants taken from newly grown shoots on 3 year-old coppiced trees all produced shoots but the capacity to do so disappeared once the coppice shoots were 4 months old. By this time the shoots had lost their juvenile leaf form and were thickened and coated with epicuticular wax.

2.12. ROOTING

In conventional propagation, it is found that cuttings from mature parts of a plant are frequently more difficult to root than those taken from juvenile

shoots. This is also observed *in vitro*, especially in shoots derived from newly-established node or shoot cultures. Thus, de Fossard *et al.,* (1977) were able to root the nodes of seedlings of several *Eucalyptus* species, but could not get any roots to form on nodes derived from mature trees. Although Sankara Rao and Venkateswara (1985) could establish shoot cultures from both juvenile and mature nodes of *Eucalyptus grandis*, 60% of the microcuttings from juvenile shoots formed roots, but only 35% rooting occurred on those from the mature source.

Although difficult, it is possible to root juvenile microshoots of *Juglans nigra* (Heile-Sudholdt *et al.,* 1986; Long *et al.,* 1995). However, when attempting to root about 300 microshoots from year-old shoot cultures from adult *J. nigra*, only one formed a root and it died during acclimatization (Pearson and Preece unpublished).

There are exceptions: shoots produced by shoot culture from juvenile or adult phases of an apple rootstock could both be rooted although their response to phloroglucinol was different (Welander and Huntrieser, 1981). Likewise, microshoots of adult and juvenile *Acer saccharinum* rooted well (Preece *et al.,* 1991).

2.13. CALLUS CULTURES

Studies on the morphology and behavior of callus illustrate the cellular basis of the maturation state. Callus cells derived from juvenile and adult phases of the same plant have different characteristics that are maintained consistently *in vitro* over prolonged periods. English ivy (*Hedera helix*) callus, derived from the juvenile stage, has been found to be composed of larger cells, to have faster growth rate, to produce roots more freely (Stoutemeyer and Britt, 1965; Polito and Alliata, 1981) and to be more prone to develop variant cell lines (Robbins and Hervey, 1970) than callus from the adult form. Callus from juvenile tissue of *Robinia pseudo-acacia* and *Castanea vulgaris* has also been noted to proliferate more rapidly than that obtained from adult zones (Trippi, 1963). Adventitious shoots could be regenerated from callus derived from seedling internodes of *Vitis vinifera* or *Vitis vinifera* x *V. rupestris*, but not from callus of similar adult explants (Rajasekaran and Mullins, 1982).

2.14. REJUVENATION INDUCED BY *IN VITRO* CULTURE

A characteristic feature of adult tissues *in vitro*, is that they become more easily managed after a period of time in culture. Most researchers consider that the marked changes that are observed in the characteristics of the cultured material indicate rejuvenation. Shoots or plantlets assume one or more of the phenotypic characteristics of plants in the juvenile phase of growth, such as a smaller leaf size and an improved capacity for adventitious root formation.

However, workers who consider juvenility to be the total reversal of maturation (e.g. Mullins, 1985; Mullins *et al.,* 1986), question whether tissue culture does really produce a juvenile state, because plantlets sometimes flower *in vitro* and plants derived from tissue cultures often flower well before those grown from seed. Therefore, some prefer the use of alternative terms such as "re-invigoration" or "apparent rejuvenation" (Pierik, 1990a,b). Because so many different characteristics of juvenility are reported in different species of plants that have been micropropagated by shoot culture, others prefer to think that a partial rejuvenation occurs, which is some of the way toward complete rejuvenation (Bonga, 1982a), and that separate features of juvenility are independently controlled.

Enhanced rooting is an indication of at least partial rejuvenation. Microshoots from adult cultures often root better than cuttings from the original donor plant. Additionally, macrocuttings from rooted microshoots often root better than from plants propagated clonally by more traditional methods. Propagators at nurseries frequently purchase micropropagated plants to serve as stock plants for cutting production. Plietzsch and Jesch (1998) compared the rooting of cuttings from conventional stock plants with those from micropropagated stock plants. They chose woody cultivars that are vegetatively propagated, not seed propagated (adult phase cultivars). For example, rooting of *Syringa vulgaris* cuttings was 0-10% from conventional stock plants and up to 85% from *in vitro*-derived stock plants and cuttings from *Prunus* 'Kanzan' rooted at 0% when harvested from conventional stock plants and up to 90% from micropropagated stock plants. However, this may be only a partial rejuvenation because *in vitro* propagated plants, rooted cuttings from *in vitro* stock plants, rooted cuttings from conventional stock plants and grafted plants of *Prunus nipponica* var. *kurilensis* 'Brillant' all flowered prolifically five years after propagation.

3. PLAGIOTROPIC GROWTH

The leading shoots of woody plants typically grow vertically upward (orthotropic growth), while lateral branches tend to grow at a more horizontal angle (plagiotropic growth). In adult angiosperms there will usually be several orthotropic shoots, but most gymnosperms usually have only one. In some New Zealand species, plagiotropy can be an adaptation that allows a plant to build up a large root system in exposed locations during the juvenile phase before tall wind-susceptible orthotropic shoots emerge (Beckett, 1979).

Although plagiotropic laterals are formed during the juvenile phase of woody plants (Fig. 11.1), in most plants an orthotropic habit of growth re-appears if such shoots are rooted as cuttings. The orthotropic habit of adult laterals can, however be persistent in some broad-leafed trees and shrubs (e.g. in *Eucalyptus cladocalyx* - Maggs and Alexander, 1967; and *Coffea* spp. - Sondahl and Sharp, 1979) and particularly in conifers. If shoots from the lateral branches of some coniferous plants are rooted as cuttings, plants are obtained where the leading shoot retains a prostrate or inclined growth habit for one or more years before eventually becoming erect. Sometimes plagiotropism appears to be permanent (e.g. in *Araucaria* spp. - Bonga, 1982; Haines and de Fossard, 1977; and in *Pseudotsuga menziesii* - Ritchie and Long, 1986). In these cases normal plants can only be obtained by rooting cuttings or grafting scions taken from vertical stems (or sometimes from juvenile shoots).

Most coniferous trees quickly gain the aspect of maturity that conveys a long-term "memory" of plagiotropism on laterals. Even though many features of juvenility can be re-established in explants by pretreatment of the stock plant material (such as plant growth regulator sprays, grafting, and repeated transfers *in vitro*), it is often found that a proportion of young trees originating from shoot multiplication *in vitro* (particularly those of conifers e.g. 40% of loblolly pine plants - Leach, 1979), have a leading shoot with a plagiotropic habit. Although in some species plagiotropism of the leading shoot often disappears after the plant has been 2-3 years in the field (Ritchie and Long, 1986), delay in shoot extension and contorted growth of the main shoot is usually unacceptable for commercial plantings. Plagiotropy imposes a severe restriction on the vegetative multiplication of plants intended for normal planting and is more likely to result from the micropropagation of adult material. This was the case in *Cunninghamia lanceolata*, where 93% of the plants obtained from shoot cultures initiated from the nodes of a juvenile clone, had orthotropic growth (which was comparable to that of seedlings), whereas only orthotropic plants were obtained from cultures initiated from the basal side shoots of an adult clone (Engelmann *et al.,* 1987). In *Sequioa sempervirens*, plagiotropic growth and poor vigour following micropropagation, are characteristics of some clones (Boulay, 1987), suggesting that there is a genetic component.

4. APICAL DOMINANCE

The primary objective with clonal micropropagation is proliferation of axillary shoots (promotion of branching). Axillary buds form from meristems in the axils of leaves. The function of axillary buds in plants is to replace the shoot apex when it is lost, or to supplement shoot apices, thus increasing the total number of leaves or the display of flowers (Napoli *et al.,* 1999). Auxins and cytokinins have important roles in regulating apical dominance. The apical region is a rich source of auxin, the supply of which can be replaced exogenously if the apex is decapitated. Similarly, exogenous applications of cytokinin will usually stimulate branching. However, when auxin and cytokinin levels are changed in intact transgenic plants, there may not always be an associated correlation with bud outgrowth (Napoli *et al.,* 1999).

Over the years, most studies on apical dominance have utilized exogenously applied plant growth regulators. Since the late 1980s, plants have been produced that have been genetically transformed to over- or under produce auxin or cytokinin (Cline, 1994), making it possible to study the effect of changed endogenous hormone levels. When plants have been transformed with an *iaaM* gene (codes for tryptophan monoxygenase), they have produced up to 10-times more IAA and had strong apical dominance and no branching. When plants have been transformed with the *iaaL* gene (codes for IAA-lysine synthetase, which inactivates IAA by causing it to

conjugate with lysine), free endogenous auxin was reduced and branching increased. When plants have been transformed with the *ipt* gene (codes for isopentenyl transferase), cytokinin content increased and the plants branched freely. Similarly, plants transformed with the *Agrobacterium rhizogenes rolC* gene (codes for cytokinin B-glucosidase, increasing free cytokinin) also had reduced apical dominance. However, when mutant pea plants have been studied, branching seems to be related to some as yet unidentified signal other than cytokinin coming from the roots (Napoli *et al.,* 1999). Therefore, the specific mechanisms of apical dominance are yet to be resolved.

Somaclonal variation is reduced when propagation is based on axillary shoot proliferation rather than adventitious regeneration, especially from callus (Preece, 1997; Preece and Trigiano, 2001). This is because axillary shoots originate from preformed meristems in leaf axils. Many commercial micropropagation labs will discard cultures that they suspect have adventitious shoots. There are many cases where nurseries have refused to purchase any more micropropagated plants because of unacceptable somaclonal variation.

To obtain axillary shoots, it is necessary that the original explant contains one or more nodes. Therefore, apical meristems, shoot tips, and stem pieces with at least one node are used as explants. To stimulate branching, cytokinins are added to the medium. The specific cytokinin and its concentration varies considerably with the genotype. Members of the Ericaceae are frequently micropropagated using isopentenyladenine (2iP). The most commonly used cytokinin for axillary shoot proliferation is benzyladenine (BA). Most species respond well to BA and when the concentration is correct, many axillary shoots will proliferate and elongate, without any adventitious shoots forming. A newer cytokinin is thidiazuron (TDZ) (Huetteman and Preece, 1993). This substituted phenylurea compound has been shown to stimulate axillary shoot growth on species where BA and 2iP are ineffective, such as *Acer saccharinum* (Preece *et al.,* 1991).

There does not appear to be a clear relationship between apical dominance in the stock plant and axillary shoot proliferation. For example, juvenile plants frequently grow more rapidly and have stronger apical dominance than adult forms. However, shoot explants from juvenile plants generally proliferate more axillary shoots than shoot explants from adult forms. Cytokinins in the medium seem to be more of a determining factor than the inherent branching habit of the chosen genotype.

There are cases where cytokinin concentrations or types are not optimal, or a species does not branch well *in vitro*. In these cases, shoots will elongate *in vitro*, but not branch. A commonly-used solution is to cut these shoots up into nodal segments and subculture them to allow for one axillary shoot to elongate per nodal explant. Although not as efficient as axillary shoot proliferation from an individual explant, this is sometime used commercially to micropropagate some plants. Automation of cutting and handling plant tissue cultures can be more efficient with a single axillary shoot elongating from each nodal explant than where masses of axillary shoots form from each explant.

5. DORMANCY

Seeds and buds of many temperate species go through a dormancy period that prevents germination or growth during times when the growing conditions are unfavorable, such as when entering winter weather conditions. By remaining dormant and not growing, plants are able to withstand unfavorable growing conditions until the weather becomes sufficiently favorable for growth. Dormancy (endodormancy) can be controlled within the seed or bud by factors such as hormones or underdeveloped structures, or by coverings such as seed coats or bud scales that may inhibit growth mechanically or chemically (Arora *et al.,* 2003, Khan 1997). Environmental factors such as unfavourable temperatures, drought, or light conditions can cause ecodormancy (also known as quiescence), which can be overcome more readily than endodormancy as environmental conditions become favourable for growth.

In his classic study, Khan (1971) demonstrated the interrelationships between gibberellins, abscisic acid (ABA), and cytokinins for maintaining or overcoming endodormancy. Gibberellins are apparently necessary for breaking dormancy. Without gibberellin, the necessary production of hydrolytic enzymes, such as α-amylase does not occur and dormancy is maintained. Even if gibberellin is present in sufficient quantity, the presence of ABA will prevent the gibberellin-induced transcription of hydrolytic enzyme genes and dormancy will be maintained. However, if both gibberellin and ABA are present in quantities

sufficient to have activity, the presence of cytokinin will overcome the inhibition of ABA and dormancy will be overcome. Numerous studies over the years have confirmed these effects of gibberellins, ABA, and cytokinins.

Recent studies on dormancy have focused on molecular biology related to onset, maintenance, and breaking of dormancy (Fennell, 1999; Arora *et al.,* 2003). These studies have utilized AFLP (amplified restriction fragment length polymorphisms), cDNA libraries, microsatellite markers, RAPD (random amplified polymorphic DNA), RFLP (restriction fragment length polymorphisms), mapping using QTL (quantitative trait loci), and inheritance studies. It appears that *Populus* bud set and outgrowth are controlled genetically and are highly variable within the genus. Howe *et al.* (1999) detected many QTLs that affect bud set and outgrowth and are testing whether specific candidate genes for endodormancy are located near these QTLs. Among these candidate genes are phytochrome genes (*PHYA-PHYE*). It makes sense to study phytochrome genes because phytochrome is the photoreceptor for red and far-red light and photoperiod (long known to be associated with inducing or overcoming dormancy in many species). Genes involved with gibberellin biosynthesis are also being studied because of the important role of gibberellins in dormancy.

Studying controlled crosses in blueberry and mapping QTLs that control the chilling requirement of buds and RAPD-based genetic linkage maps, Rowland *et al.* (1999) have developed a two-gene model that predicts observed segregation ratios for the chilling requirement. In their model, it is assumed that the genes have equal and additive effects. Cloning and mapping these genes will allow the determination of whether they are associated with the mapped QTL loci that control the chilling requirement of blueberry.

Generally, cultures are most easily established from explants harvested from the active growth of the mother plant. The best time for collecting explants is usually when stock plants are producing new vigorous shoots (i.e. in temperate regions, in spring to early summer), but many exceptions have been observed. The optimum time for collecting explants may depend on the kind of culture, on the particular genotype to be used, on the relative amounts of contamination, and browning of the explant occurring at each season.

A major detriment to using dormant buds as explants is the occurrence of microbes within the buds. The outer scales of dormant buds of *Acer*, *Aesculus*, *Betula*, *Fagus, Populus*, *Quercus*, and *Ulmus* were shown to host populations of fungi and bacteria (Warren, 1976). Additionally, microbes were found in the internal parts of *Tilia* and *Fraxinus* buds. Although various treatments were tested to clean up dormant buds of *Fraxinus americana*, there was still 100% microbial contamination of cultures (Preece and others unpublished). However, isolated apical meristems from such buds can be disinfested of microorganisms without killing them.

Nevertheless, it is possible to obtain clean cultures from dormant buds of some species, and because of their resting state, they have been tolerant of harsher disinfestation treatments. In fact, buds can be dipped in alcohol and flamed with no serious injury (Bonga and Von Aderkas, 1992). Typically, actively growing explants are surface disinfested only once using 0.5-1.0% hypochlorite solution with a wetting agent for up to 30 minutes, followed by rinses of sterile distilled water. Sometimes there will be a short dip in 70% alcohol prior to the hypochlorite treatment. Using stem segments with dormant axillary buds of *Pyrus calleryana*, Rossi *et al.* (1991) used a concentration of 3% sodium hypochlorite for 20 minutes and only had a 20% rate of contamination. Shevade and Preece (1993) used a double-disinfestation technique for flower buds of *Rhododendron* collected from field-grown plants during the winter from November through February. They disinfested the entire buds for 15 minutes in 0.5% NaOCl containing Tween 20. This was followed by rinses in sterile water and removal of the outer scales under aseptic conditions to expose the thin white scales surrounding the florets. They were then surface disinfested and rinsed again using the same method as applied to the whole buds. *Prunus serotina* (black cherry) buds could be collected beginning in February, but were washed in running tap water for an hour, followed by a benomyl (fungicide) soak for 10 minutes, then 20 minutes in 0.5% NaClO and rinsing (Maynard *et al.,* 1991). Bud scales were then removed aseptically and the bud was placed in concentrated hydrogen peroxide for 10-30 seconds, then rinsed.

There have been cases where explants were difficult to establish *in vitro* or growth has been inadequate if they were excised from dormant plants. Norton and Norton (1989) had difficulty with establishment if *Rhododendron* shoots were cultured initially in the winter or when the stock plant was not actively growing. The proportion of *Prunus*

'Accolade' meristems that gave rise to established shoots with a rosette of leaves progressively increased in shoots excised from field-grown trees early December (winter) to May (Quoirin *et al.,* 1974). Potato buds excised in spring or early summer rooted better than those taken later in the year (Mellor and Stace-Smith, 1969), and a greater proportion of *Castanea sativa* cuttings produced callus *in vitro* if excised and cultured in March (spring) rather than in December (Borrod, 1971). However, in a *Castanea* hybrid, the proportion of December-gathered cuttings forming callus was high, and the poorest rate of culture establishment occurred with explants taken in July.

5.1. EXCEPTIONS TO THE GENERAL RULE

Although the best time for sampling usually coincides with the period when stock plants are in active growth, this is not always the case. Bulblet initiation *in vitro* from bulbs of *Tulipa* 'Hageri' was best in May, when the mother bulbs were growing vigorously, but in 'Apeldoorn' it was best in August, at harvest time (Nishiuchi, 1979). Similarly sections of the immature floral stems of *Tulipa* 'Merry Widow' bulbs only gave rise to direct adventitious buds when explants were taken from dormant bulbs (Wright and Alderson, 1980). Once the bulbs had entered a stage of active growth, the capacity to produce shoots practically disappeared. Callus continued to be formed, but it too was not produced once the floral stem had begun active extension growth. The formation of adventitious buds from cylinders cut from the base of scale leaves of *Lilium speciosum* was high when explants were taken during spring and autumn, but practically nil in summer and winter (Robb, 1957).

5.2. PLANTS IN GREENHOUSES AND GROWTH ROOMS

Explants are primarily taken from actively growing shoots from plants grown in protected environments. There are tiny cracks and crevasses in plants in which fungal spores and bacteria can exist. It can be extremely difficult to get a surface disinfestant into these areas. Therefore, success with disinfestation is less from field-grown stock plants than those grown in the greenhouse, growth chamber or other protected environment. It is likely that the rain and blowing dust that strike field-grown plants carry or create an environment conducive to microorganisms.

Simply because a plant is grown in a greenhouse or growth room does not mean that it is free from surface microbes. The cultural practices by which the plant is grown are important. For example, it is essential that water be kept off the above-ground parts of the plant and that care be taken to water only the soil or growing medium.

5.3. FORCING SHOOTS OF TEMPERATE WOODY PERENNIALS

Many woody plants are either too large or are growing in the ground and cannot be moved in a practical sense to a greenhouse or growth room. In temperate regions, it is therefore often necessary to remove branch tips or segments, usually during the dormant season, and force new shoot growth on them indoors or in a greenhouse environment. This new growth can then be excised, surface disinfested and used for explant material. Although there are species differences, these explants tend to be relatively contaminant-free, they may also be more readily established in culture than explants taken during spring and summer, and can be less prone to produce dark, polyphenolic exudates.

There is variation in the technique among different labs. A common method is to excise 20-25 cm long stems (Read and Yang, 1991) from deciduous trees and shrubs, soak for 15 minutes in bleach solution containing 0.78% NaOCl with Tween 20 (Read and Yang, 1988, 1991; Yang and Read, 1992, 1993). The bleach disinfestation enhances bud break compared to non-disinfested stem tips (Read and Preece, 2003), in fact Yang and Read (1992) reported that buds broke faster, more buds elongated, and shoot elongation was promoted if the stems received the 15 minute soak in bleach solution prior to forcing, compared to those that did not. There is a fresh cut made at the base and the basal portions of the stems are placed in containers with water, 200 mg/l of the commonly used cut flower preservative 8-hydroxyquinoline citrate (8-HQC) (Read and Yang, 1987), sucrose, silver thiosulphate and sometimes plant growth regulators (Read and Preece, 2003). The influence of the plant growth regulators is discussed below under stock plant treatments.

Arrillaga *et al.* (1991) excised leafless shoots in April from a 30 year-old *Sorbus domestica* tree, placed them in jars with a 10% benomyl solution and sprayed them with a 44.4µM solution of BA to encourage lateral growth. They harvested shoots for *in vitro* culture after 10-15 days. The resulting shoots grew *in vitro* in a manner that was similar to juvenile source explants.

A related technique is harvesting 3-4 cm long terminal, lignified stem sections, immersing the cut ends in plant growth regulator solutions, placing in a greenhouse medium, and forcing in the greenhouse under a 24 hour photoperiod, as Onay (2000) did with 30 year-old pistachio. New shoots grew and were sufficiently large to excise, surface disinfest and place *in vitro*.

There may be two main disadvantages of forcing new growth from shoot tips taken from adult woody plants. One is that these shoots are excised from the most adult portions of the plant, which does not readily allow for rejuvenation. Additionally, with some recalcitrant species, shoots will elongate, but rapidly decline before being sufficiently long for excision. This has been the case with *Juglans nigra* (Eastern black walnut), where the apical buds elongated, but wilted and declined when they were 1-2 cm long (Khan and Preece, unpublished). This did not seem to relate to month of collection. However, Read and Yang (1987) harvested stems from *Castanea dentata* (American chestnut) in late November and stored them for various times at 4.5°C. Buds did not grow well if stored 45 days or less but grew well and elongated if stored 75 days or longer, with the best elongation on stems stored 105 days.

A method to force shoots from within the cone of juvenility is to use larger stem segments taken from the bases of the larger branches growing from the trunk of a tree or the base of a shrub (Henry and Preece 1997 a, b; Preece *et al.,* 2002; Van Sambeek *et al.,* 1997 a, b; Van Sambeek and Preece, 1999). Vieitez *et al.* (1994) compared explant establishment from leafy shoots that flushed from old stem segments, from the previous year's growth, and from the current season's growth of 100 year-old *Quercus robur* trees. They were unsuccessful in establishing shoot cultures from the current season's growth or from the previous year's growth, but were successful with explants from new shoots that grew on old stem segments. Using the old stem segments, they were able to establish shoot cultures from oak trees ranging from 70-300 years-old.

6. ADDITIONAL STOCK PLANT TREATMENTS

The ontogeny and physiology of the plant that is the source of explants (stock plants or mother plants) has a profound influence on *in vitro* response. Results obtained *in vitro* can be influenced by the condition of the stock plants, the ways that they are treated and by the environment in which they have been grown. It is generally recognized that the best explant material is derived from healthy and vigorous plants that have been maintained in active growth without stress. There are additional factors that will also have an effect.

6.1. NUTRITION

Explants that are most suitable for *in vitro* culture, are generally obtained from stock plants that have received adequate mineral nutrition (Read and Preece, 2003). Barker *et al.* (1977) could culture adult nodes of *Eucalyptus* most successfully if they were excised from healthy well-watered and well-fertilized trees. Debergh and Maene (1985) found that treating *Ficus lyrata* stock plants with high levels of Mg resulted in increased shoot formation from leaf explants. However, N, P, and K had no influence and high levels of Ca reduced shoot formation. Explants of papaya were more responsive to *in vitro* culture if the mother plant had been given fertilizer and water consistently in the field (Litz and Conover, 1977). Tomato stock plant nitrogen nutrition had an influence on adventitious shoot production from leaf explants, with higher levels of nitrogen resulting in more shoots (Read *et al.,* 1979). Later work (Read and Economou, 1987) showed that supplying tomato stock plants with either very low or very high rates of inorganic nitrogen reduced the number of shoots that could be regenerated from cultured leaf segments. Nitrogen and potassium nutrition were also found to affect the results obtained from tissue culturing several *Salix* clones.

6.2. DISEASE STATUS

Usually the presence of any kind of plant disease is a reason for rejecting mother plants, because infectious organisms are liable to cause the death of cultures, may have a debilitating and negative effect on cultures, or may be transmitted to propagated plants. Greno *et al.* (1988) found that shoot cultures of several *Citrus* species were affected by the presence of the virus and virus-like agents: Citrus tristeza virus (CTV), Citrus infectious variegation virus (CIVV), Psorosis Veinenation, Citrus cachexia viroid (CcaV), and Citrus exocortis viroid complex. In general, infection of the mother plant reduced the number and size of shoots that developed. Similarly, the production of axillary shoots, and the number of plants finally established, tended to be lower in infected cultures than in healthy ones. The actual

responses obtained varied both according to the host plant and the pathogen. In some hosts a pathogen might have no effect or may not cause visible symptoms. Greno *et al.* (1988) have suggested that the presence of such latent virus infections could be a cause of the difficulties in defining standard micropropagation protocols for some perennial plants and of variation among the resulting plants.

6.3. PRUNING

Cutting back stock plants to induce the growth of juvenile shoots was described earlier in this chapter. When initiating shoot cultures, it can also be advantageous to prune old herbaceous or woody plants to obtain a flush of new shoots bearing young, smaller and less contaminated buds (Read, 1988). Pruning of stock plants is also used to time flushes of growth, to increase the number of shoots that can be harvested (speeds collection), and for stock plant management (Scott, 1987). Another result of pruning stock plants can be to obtain explants of a more desirable size. Debergh and Maene (1985) reported that axillary buds of different members of the Araceae are too large or well-developed to be placed *in vitro*, whereas new shoots that grow following pruning have smaller buds that are easier to put in culture. Preece (1987) reported that decapitation of tomato stock plants decreased rooting and dry weight production of leaf explants *in vitro* when they were excised from the stock plant up to 8 days after pruning.

6.4. LIGHTING

Changes in stock plants can be brought about by the photon flux of light, its wavelength, or photoperiod (day length). These can also affect the growth or morphogenesis of cultured explants.

6.4.1. Photon flux

Growth in 22°C and 12 h of high light was found to be the most effective stock plant treatment for obtaining growth and shoot formation of single node explants of *Eucalyptus* (Cresswell and Nitsch, 1975). However, tomato stock plants grown under reduced light gave the highest yield of tomato protoplasts (Cassells and Barlass, 1978).

Pelargonium petioles formed the highest numbers of roots in response to nitrogen and sucrose in an *in vitro* medium when they were derived from stock plants that had been maintained under an irradiance of 2.5 rather than 11.6 or 23.0 W m^{-2} (Welander, 1978). Read and Economou (1987) found that microcuttings from azalea stock plants irradiated with low levels of light rooted better than if the stock plants received a high photon flux. Rooting of macro-cuttings can be enhanced by or inhibited by collecting cuttings from stock plants that have been grown under high levels of light (Hansen, 1987). The rooting response seems to be species specific.

6.4.2. Wavelength

The branching of intact *Petunia hybrida* plants can be modified through the phytochrome system. Plants grown in 10 hours of white light, terminated by 30 minutes red light each day become short and branched, but when the light period is terminated by 30 minutes far-red light, tall single-stemmed plants are obtained (Read *et al.,* 1978; Suriyajantratong, 1979). Leaf discs cut from stock plants receiving red light tended to produce a greater fresh- and dry-weight of callus and a larger number of adventitious shoots than those removed from stock plants receiving far-red light. The differences in growth and differentiation *in vitro* were probably related to altered natural growth substance levels in the two groups of plants. Extractable free IAA (auxin) was higher in plants treated with far-red light than those receiving red light.

6.4.3. Photoperiod

Hilding and Welander (1976) found that the most effective combination of auxin and cytokinin to induce morphogenesis in petiole explants of *Begonia hiemalis* varied according to whether stock plants had been grown under long or short days. Leaf petioles of this species only produced adventitious shoots and roots if they were taken from plants grown in 15-16 hour days at 18-20°C. There was no organogenesis on those taken from plants grown in short days (7-8 hours at 15°C) (Welander, 1977). Similar results were obtained on the same species by Simmonds and Nelson (1989). In contrast, Dunwell and Perry (1973) found that anthers taken from *Nicotiana tabacum* stock plants kept in an 8 h high irradiance photoperiod, produced the greatest numbers of haploid plants from cultured anthers.

6.5. TEMPERATURE

Leaf discs of *Streptocarpus* produced more shoots and roots when mother plants were grown at 12°C rather than 18°C or 24°C (Appelgren and Heide, 1972), and explants from *Begonia hiemalis* stock plants maintained in growth rooms, formed the most shoots and roots when the growth room temperature was 15°C rather than 18°C or 21°C (Appelgren,

1976). When greenhouse-grown plants were used as a source, the optimum cultural temperature for morphogenesis varied according to the time of year at which explants were removed. In October, the optimum temperature was 18°C, in December, it was 15°C, and it was 21°C in February.

The temperature and length of time at which bulbs have been stored can affect the subsequent production of adventitious bulblets *in vitro*. Scale explants from *Lilium longiflorum* bulbs produced a smaller number of larger bulblets if they had been taken from bulbs stored for less than 110 days, compared to those taken from bulbs stored for a longer period (Stimart and Ascher, 1981b). An effect of storage temperature on subsequent bulblet size has also been demonstrated in *Hyacinthus*. In addition, explants from bulbs kept at 5°C for 35 days, produced a larger number of bulblets than those taken from bulbs stored for a longer period at this temperature (Ko, 1986: reported in Read, 1988).

Onion bulbs were planted in a greenhouse and grown at 18°C (Puddephat *et al.,* 1999). When they began to bolt, they were moved to growth chambers at either 10 or 15°C. Embryogenic cultures were established from nonpollinated ovules. When the stock plants were maintained at 15°C, there was a ten-fold increase in embryogenesis compared to when the stock plants were maintained at 10°C.

6.6. PLANT GROWTH REGULATOR PRE-TREATMENTS

Treating the stock plant with plant growth regulators can have an influence on *in vitro* performance of explants (Preece, 1987). As explained above, sprays of gibberellin and/or cytokinin are often effective in introducing some degree of re-juvenation (or re-invigoration) of woody plants, enabling explants to be cultured where previously it was impossible, and frequently inducing axillary bud break and increasing the number of shoots from which explants can be taken.

A convenient method of applying plant growth regulators is to incorporate them into the forcing solution when leafless branches are taken from deciduous woody plants, or to apply them as a soak before forcing bud growth. Read and Yang (1987) report that shoot proliferation from shoot tips of *Philadelphus* and *Dirca* was increased by introducing BA into the solution into which twigs were placed for forcing in early spring. Yang and Read (1993) forced dormant stems of Vanhoutte's spirea in solutions containing BA or GA_3. A higher percentage of explants produced shoots or more shoots were produced per explant if the forcing solution contained 44.4 µM compared to 4.4 µM BA. However, as GA_3 concentration in the forcing solution increased from 2.9 to 145 µM, the number of explants that produced shoots decreased. When GA_3 was in the forcing solution of American chestnut or *Aesculus*, there was no effect on *in vitro* performance of shoot explants. Preece (1987) and Preece *et al.* (1984) reported that pretreating stock plants with gibberellic acid can inhibit adventitious shoot formation and rooting when tomato leaf explants are subsequently cultured with BA.

When lignified stems were excised from 30 year-old *Pistacia vera* trees, the bases were treated with either 44µM BA or 49 µM IBA for 24 hours (Onay, 2000). The stems were then potted up and placed in a greenhouse. Shoots only grew from stems treated with BA and these established *in vitro* and microshoots proliferated. Donkers and Evers (1987) obtained a significant increase in the multiplication rate from certain buds if flushed shoots of *Platanus acerifolia* had been sprayed with 200 µM BA in 50% ethanol.

Adventitious shoot formation can also be influenced by cytokinin pre-treatments. Economou and Read (1980) discovered that if leaves of *Petunia* were dipped for 30 seconds into a solution of 1.78 mM BA, this allowed adventitious shoots to develop freely when segments of the leaves were later cultured on a cytokinin-free medium. Oka and Ohyama (1981) obtained adventitious bud formation from mulberry leaf explants when seedling mother plants or *in vitro* shoots were pre-treated with BA.

De Langhe and de Bruijne (1976) found that the shoot forming ability of cultured shoot explants of tomato was stimulated when the parental plants had been previously pre-treated with chlormequat chloride, but that if this compound was added instead to the culture medium, it was ineffective. This observation was confirmed by Read *et al.* (1978, 1979). It was also observed that shoots excised from *Dahlia* leaves gave rise to increased amounts of callus if the mother plants had been grown in short days or had been sprayed with a 2500 mg/l daminozide solution (Gavinlertvatana *et al.,* 1979).

Pre-treating wheat plants with sprays of 2,4-D and several other herbicides during the first week of embryo development caused multiple shoot formation from the embryos at a later stage (Ferguson and McEwan, 1970). This appears to occur through the *in vivo* formation of adventitious embryos (Ferguson *et al.,* 1979).

7. CONCLUSIONS

Most *in vitro* studies focus on manipulating the medium. Indeed this can lead to profound effects on explant growth and development. However, not all explants respond well *in vitro*. Success can be increased by considering the plants that are the sources of explants. Simply the time of year or portion of the stock plant from which explants are collected can have great influences on *in vitro* performance. Additionally, it may be necessary to rejuvenate or otherwise treat the stock plant or donor tissues prior to harvesting explants to place in culture.

REFERENCES

ABO EL-NIL M. 1982 Method for asexual reproduction of coniferous trees. U.S. Patent No. 4353184.

APPELGREN M. 1976 Regeneration of *Begonia hiemalis in vitro*. Acta Hortic. 64, 31-38.

APPELGREN M. & HEIDE O. 1972 Regeneration in *Streptocarpus* leaf discs and its regulation by temperature and growth substances. Physiol. Plant. 27, 417-423.

ARORA, R., ROWLAND L.J. & TANINO K. 2003 Inductionand release of bud dormancy in woody perennials: ascience comes of age. HortScience 38:911-921.

ARRILLAGA I., MARZO T. & SEGURA J. 1991 Micropropagation of juvenile and adult *Sorbus domestica* L. Plant Cell Tissue Organ Cult. 27, 341-348.

BABU K.N., ANU A., REMASHREE A.B. & PRAVEEN K. 2000 Micropropagation of curry leaf tree. Plant Cell Tissue Organ Cult. 61, 199-203.

BALLESTER A., SANCHEZ M.C & VIEITEZ A.M. 1989 Etiolation as a pretreatment for *in vitro* establishment and multiplication of sweet chestnut. Physiol. Plant. 77, 395-400.

BARGHCHI M. 1986 *In vitro* culture of mature commercial *Pistacia vera* L. cultivars. Comb. Proc. Int. Plant Prop. Soc. 1985 35, 331-333.

BARKER P.K. DE FOSSARD R.A. & BOURNE R.A. 1977 Progress towards clonal propagation of *Eucalyptus* species by using culture techniques. Comb. Proc. Int. Plant Prop. Soc. 27, 546-556.

BECKETT K.A. 1979 Juvenility with a special reference to New Zealand plants. The Plantsman 1, 81-84.

BENNETT L.K. & DAVIES F.T. 1986 *In vitro* propagation of *Quercus shumardii* seedlings. HortScience 21, 1045-1047.

BHOJWANI S.S., MULLINS K. &. COHEN D. 1987 Micropropagation of *Feijoa sellowiana* Berg. Acta Hortic. 212, 69-73.

BLACK D.K. 1972 The influence of shoot origin on the rooting of douglas-fir stem cuttings. Comb. Proc. Intl. Plant Prop. Soc. 22, 142-159.

BONGA J.M. 1982 Vegetative propagation in relation to juvenility, maturity and rejuvenation. pp. 387-412 *in* Bonga J.M. and Durzan D.J. (eds.) *Tissue Culture in Forestry*. Martinus Nijhoff, Dordrecht.

BONGA J.M. & VON ADERKAS P. 1992 *In vitro* culture of trees. Kluwer, Dordrecht. 236 pp.

BORROD G. 1971. Contribution à l'étude des cultures *in vitro* des tissus de chataignier. Compt. Rend. Acad. Sci. Paris 272D, 56-59.

BOULAY M. 1987 *In vitro* propagation of tree species. pp. 367-382 *in* Green C.E., Somers, D.A., Hackett W.P. and Biesboer D.D. (eds.) *Plant Tissue and Cell Culture*. Alan R. Liss, Inc., New York.

BRINK R.A. 1962 Phase change in higher plants and somatic cell heredity. Q. Rev. Biol. 37, 1-22.

CASSELLS A.C. & BARLASS M. 1978 A method for the isolation of stable mesophyll protoplasts from tomato leaves throughout the year under standard conditions. Physiol. Plant. 42, 236-242.

CHEVRE A.-M. & SALESSES G. 1987 Choice of explants for chestnut micropropagation. Acta Hortic. 212, 517-523.

CHRISTIANSON M.L. 1987 Causal effects in morphogenesis. pp. 45-55 *in* Green C.E., Somers, D.A., Hackett, W.P.& Biesboer D.D. (eds.) *Plant Cell and Tissue Culture.* Alan R. Liss, Inc. New York.

CHRISTIANSON M.L. & WARNICK D.A. 1983 Competence and determination in the process of *in vitro* shoot organogenesis. Dev. Biol. 95, 288-293.

CHRISTIANSON M.L. & WARNICK D.A. 1988 Organogenesis *in vitro* as a developmental process. HortScience 23, 515-519.

CLARK J.R. 1982 Juvenility and plant propagation. Comb. Proc. Int. Plant Prop. Soc. 1981 31, 449-453.

CLINE M.G. 1994 The role of hormones in apical dominance. New approaches to an old problem in plant development. Physiol. Plant. 90, 230-237.

CRESSWELL R. & NITSCH C. 1975 Organ culture of *Eucalyptus grandis*. Planta 125, 87-90.

DE FOSSARD R.A., BARKER P.K. & BOURNE R.A. 1977 The organ culture of nodes of four species of *Eucalyptus*. Acta Hortic. 78, 157-165.

DE LANGHE E. & DE BRUIJNE E. 1976 Continuous propagation of tomato plants by means of callus cultures. Sci. Hortic. 4, 221-227.

DEBERGH P. & MAENE L. 1985 Some aspects of stock-plant preparation for tissue culture propagation. Acta Hortic. 166, 21-23.

DENNIN K.A. & McDANIEL C.N. 1985 Floral determination in axillary buds of *Nicotiana silvestris*. Dev. Biol. 112, 377-382.

DONKERS J. & EVERS P. 1987 Growth regulator preconditioning of *Platanus acerifolia* trees for micropropagation. Acta Hortic. 212, 113-116.

DUNWELL J.M. & PERRY M.E. 1973 The influence of *in vivo* growth conditions of *N. tabacum* plants on the *in vitro* embryogenic potential of their anthers. Annu. Rep. John Innes Inst. 64:69-70.

DURAND-CRESSWELL R., BOULAY M. & FRANCLET A. 1982 Vegetative propagation of *Eucalyptus*. pp.150-181 *in* Bonga J.M. and Durzan D.J. (eds.) *Tissue Culture in Forestry*. Martinus Nijhoff, Dordrecht.

ECONOMOU A. & READ P.E. 1980 Effect of benzyladenine pretreatments on shoot proliferation from petunia leaf segments. Proc. Plant Growth Reg. Working Group, U.S.A. 7:96-103.

ENGELMANN F., GODIN G., LEBOEUF J. & BIGOT C. 1987 *In vitro* propagation of juvenile and adult clones of *Cunninghamia lanceolata* (Lamb.) Hook. Acta Hortic. 212, 479-487.

FENNELL A. 1999 Systems and approaches to studying dormancy: Introduction to the workshop. HortScience 24, 1172-1173.

FERGUSON J.D. & McEWAN J.M. 1970 The chemical induction of supernumerary shoots in the developing embryos of wheat. Physiol. Plant. 23, 18-28.

FERGUSON J.D., McEWAN J.M. & CARD K.A. 1979 Hormonally-induced polyembryos in wheat. Physiol. Plant. 45, 470-474.

FISHEL D.W., ZACZEK J.J. & PREECE J.E. 2003 Positional influence on rooting of shoots forced from the main bole in swamp white oak and northern red oak. Can. J. Forest Res. 33, 705-711.

FISHER F.J.F. 1954 Effect of temperature on leaf-shape in *Ranunculus*. Nature 173, 406-407.

FRANCLET A. 1987 Expos introductif. pp. 23-40 *in* Ducaté, G., Jacob M. and Simeon A. (eds.). *Plant Micropropagation in Horticultural Industries. Preparation, Hardening and Acclimatization Processes*. Belgian Plant Tissue Culture Group, Symposium Florizel 87; Arlon, Belgium.

GAVINLERTVATANA P., READ V., WILKINS H.F. & HEINS R. 1979 Influence of photoperiod and daminozide stock plant pretreatments on ethylene and CO_2 levels and callus formation from *Dahlia* leaf segment cultures. J. Am. Soc. Hort. Sci. 104: 849-852.

GENEVE R.L., HACKETT W.P. & SWANSON B.T. 1988 Adventitious root initiation in de-bladed petioles from the juvenile and mature phases of English Ivy. J. Am. Soc. Hort. Sci. 113:630-635.

GEORGE L.J. & PREECE J.E. 2004 The influence of location of main stem segment origin on forcing and rooting of softwood shoots of *Betula nigra* L. HortScience 39, 890.

GIOVANNELLI A. & GIANNINI R. 1999 Effect fo serial grafting on micropropagation of chestnut (*Castanea sativa* Mill.). Acta Hortic. 494, 243-245.

GRENO V., NAVARRO L., DURAN-VILAN N. 1988 Influence of virus and virus-like agents on the development of citrus buds cultured *in vitro*. Plant Cell Tissue Organ Cult. 15, 113-124.

HACKETT W. P. 1987 Juvenility and maturity. pp. 216-231 in Bonga J. M.,& Durzan D. J., (eds.) *Cell and Tissue Culture in Forestry, Vol. 3. Case histories: Gymnosperms, Angiosperms and Palms.* Martinus-Nijhoff Publishers, Dordrecht, Boston, Lancaster. ISBN 90-247-3432-0.

HACKETT W.P. & MURRAY J.R. 1997 Approaches to understanding maturation or phase change. pp. 73-86. *in* Geneve, R.L, Preece J.E. and Merkle S.A. (eds.) *Biotechnology of Orna-mental Plants.* CAB International, Wallingord, UK.

HACKETT W.P. 1976 Control of phase change in woody plants. Acta Hortic. 56, 143-154.

HACKETT W.P. 1985 Juvenility, maturation and rejuvenation in woody plants. Hort. Rev. 7, 109-155.

HAINES R.J. & DE FOSSARD R.A. 1977 Propagation of Hoop pine (*Araucaria cunninghamii*) by organ culture. Acta Hortic. 78, 297-301.

HANSEN J. 1987 Stock plant lighting and adventitious root formation. HortScience 22, 746-749.

HANSEN K.C. & LAZARTE J.E. 1984 *In vitro* propagation of pecan seedlings. HortScience 19, 237-239.

HANUS D. & ROHR R. 1987 *In vitro* plantlet regeneration from juvenile and mature sycamore maple. Acta Hortic. 212, 77-82.

HARTMANN H. T., KESTER D.E., DAVIES F.T. Jr. & GENEVE R.L. 2002 *Hartmann and Kester's Plant Propagation Principles and Practices. 7th ed.* Prentice-Hall, Upper Saddle River, N.J. 880 pp.

HEILE-SUDHOLT C., HUETTEMAN C.A., PREECE J.E., VAN SAMBEEK J.W. & GAFFNEY G.R. 1986 *In vitro* embryonic axes and seedling shoot tip culture of *Juglans nigra* L. Plant Cell Tissue Organ Cult. 6, 189-197.

HENRY P.H. & PREECE J.E. 1997a Production and rooting of shoots generated from dormant stem sections of maple species. HortScience 32, 1274-1275.

HENRY P.H. & PREECE J.E. 1997b Production of shoots from dormant *Acer* as influenced by length and caliper of stem sections. J. Environ. Hort. 15, 153-156.

HILDING A. & WELANDER T. 1976 Effects of some factors on propagation of *Begonia hiemalis in vitro*. Swed. J. Agr. Res. 6, 191-199.

HOWARD B. 1990 Rooting potential in cuttings. The Garden 115, 608-611.

HOWE G.T., DAVIS J., JEKNIC Z., CHEN T.H.H., FREWEN B., BRADSHAW H. D. Jr. & SARUUL P. 1999 Physiological and genetic approaches to studying endodormancy-related traits in *Populus*. HortScience 34, 1174-1184

HUDSON J.P. & WILLIAMS I.H. 1955 Juvenility phenomena associated with crown gall. Nature 175, 814.

HUETTEMAN C.A. & PREECE J.E. 1993 Thidiazuron: A potent cytokinin for woody plant tissue culture. Plant Cell Tissue Organ Cult. 33, 105-119.

JAMES S. 1984 Lignotubers and burls – their structure, function and ecological significance in Mediterranean ecosystems. Bot. Rev. 50, 225-256.

JORGENSEN J. 1989 Somatic embryogenesis in *Aesculus hippocastanum* L. by culture of filament callus. J. Plant Physiol. 135, 240-241.

KHAN A.A. 1971 Cytokinins: Permissive role in seed germination. Science. 171, 853-859.

KHAN A.A. 1997 Quantification of plant dormancy: Introduction to the workshop. HortScience 32, 608-609.

LEACH G.N. 1979 Growth in soil of plantlets produced by tissue culture - loblolly pine. Tappi 62, 59-61.

LEDBETTER D.I. & PREECE J.E. 2003 Forcing softwood shoots, rooting, and micropropagating *Hydrangea quercifolia* Bartr. Prop. Orn. Plants 3, 3-10.

LEE S.K. & RAO A.N. 1986 *In vitro* regeneration of plantlets in *Fagraea fragrans* Roxb. - a tropical tree. Plant Cell Tissue Organ Cult. 7, 43-51.

LIBBY W.J. & HOOD J.V. 1976 Juvenility in hedged radiata pine. Acta Hortic. 56, 91-98.

LITZ R.E. & CONOVER R.A. 1977 Tissue culture propagation of some foliage plants. Proc. Fla. State Hort. Soc. 90, 301-303.

LONG L.M., PREECE J.E. & VAN SAMBEEK J.W. 1995. Adventitious regeneration of *Juglans nigra* L. (Eastern black walnut). Plant Cell Rep. 14, 799-803.

MAGGS D.H. & ALEXANDER D. M. 1967 A topophysic relation between regrowth and pruning in *Eucalyptus cladocalyx* F. Muell. Aust. J. Bot. 15, 1-9.

MAYNARD C.A., KAVANAGH K., FUERNKRANZ H. & DREW A.P. 1991 Black Cherry (*Prunus serotina* Ehrh.). pp. 3-22 *in* Bajaj Y.P.S. (ed.) *Biotechnology in Agriculture and Forestry, Vol. 16, Trees III,* Springer Verlag, Berlin.

McCOWN B.H. 1989. Birch (*Betula* spp.). *in* Bajaj, Y.P.S. (ed.). *Biotechnology in Agriculture and Forestry, Vol. 5, Trees II.* Springer Verlag, Berlin.

McGRANAHAN G., LESLIE C.A. & DRIVER J.A. 1988 *In vitro* propagation of mature Persian walnut cultivars. HortScience 23, 220.

MELLOR F.C. & STACE-SMITH R. 1969 Development of excised potato buds in nutrient medium. Can. J. Bot. 47, 1617-1621.

MEYER M.M. Jr. 1984. Clonal propagation of perennial plants from flowers by tissue culture. Comb. Proc. Int. Plant Prop. Soc. 1983 33, 402-407.

MOHAMMED G.H. & VIDAVER W.E. 1988 Root production and plantlet development in tissue-cultured conifers. Plant Cell Tissue Organ Cult. 14, 137-160.

MONTEUUIS O., BON M.C. & BERTHON J.Y. 1987 Micropropagation aspects of *Sequoiadendron giganteum* juvenile and mature clones. Acta Hortic. 212, 489-497.

MULLINS M.G. 1985 Regulation of adventitious root formation in microcuttings. Acta Hortic. 166, 53-61.

MULLINS M.G., SRISKANDARAJAH S. & NOITON D. 1986 Enhanced capacity for adventitious root formation in apple microcuttings by conditioning of mother cuttings. Acta Hortic. 179, 873-874.

NAPOLI C.A., BEVERIDGE C.A. & SNOWDEN K.C. 1999 Reevaluating concepts of apical dominance and the control of axillary bud outgrowth. Curr. Top. Dev. Biol. 44, 127-167.

NISHIUCHI Y. 1979. Studies on the vegetative propagation of tulip. II. Formation and development of adventitious buds on excised scales cultured *in vitro*. J. Jap. Soc. Hort. Sci. 48:99-105.

NORTON C.R. & NORTON M.E. 1989 Rhododendrons. pp 428-451 *in* Bajaj, Y.P.S. (ed.) *Biotechnology in Agriculture and Forestry, Vol. 5, Trees II*. Springer-Verlag, Berlin.

OKA S. & OHYAMA K. 1981 *In vitro* initiation of adventitious buds and modification by high concentrations of benzyladenine in leaf tissues of mulberry (*Morus alba*). Can. J. Bot. 59, 68-74.

ONAY A. 2000 Micropropagation of Pistacio from mature trees. Plant Cell Tissue Organ Cult. 60, 159-162.

PEREZ-PARRON M.A., GONZALEZ-BENITO M.E. & PEREZ C. 1994 Micropropagation of *Fraxinus angustifolia* from mature and juvenile plant material. Plant Cell Tissue Organ Cult. 37, 297-302.

PEVALEK-KOZLINA B. & JELASKA S. 1987 Microclonal propagation of *Prunus avium* L. Acta Hortic. 212, 599-602.

PHARIS R.P. & KUO C.G. 1977 Physiology of gibberellins in conifers. Can. J. Forest Res. 7, 299-325.

PHARIS R.P. & KING R.W. 1985 Gibberellins and reproductive development in seed plants. Annu. Rev. Plant Physiol. 36, 517-568.

PHARIS R.P., ROSS S.D. & McMULLAN E. 1980 Promotion of flowering in the Pinaceae by gibberellins. Physiol. Plant. 50, 119-126.

PHARIS R.P., ROSS S.D., WAMPLE R.L. & OWENS J.N. 1976 Promotion of flowering in conifers of the Pinaceae by certain of the gibberellins. Acta Hortic. 56, 155-162.

PIERIK R.L.M. 1990a. Rejuvenation and micropropagation. pp. 91-101 *in* Nijkamp, H.J.J., Van Der Plas L.H.W. and Van Aartrijk J. (eds.) *Progress in Plant Cellular and Molecular Biology*. Proc. VIIth Int. Cong. on Plant Tissue & Cell Culture. Amsterdam, The Netherlands. Kluwer, Dortrecht.

PIERIK R.L.M. 1990b. Rejuvenation and micropropagation. Newsl. Int. Assoc. Plant Tiss. Cult. 62, 11-21.

PLIEGO-ALFARO F. & MURASHIGE T. 1987 Possible rejuvenation of adult avocado by graftage onto juvenile rootstocks *in vitro*. HorstScience 22, 1321-1324.

PLIETZSCH A. & JESCH H.-H. 1998 Using *in vitro* propagation to rejuvenate difficult-to-root woody plants. Comb. Proc. Intl. Plant Prop. Soc. 48:171-176.

POETHIG R.S. 2003 Phase change and the regulation of development timing in plants. Science 301, 334-336.

POLITO V.S. & ALLIATA V. 1981 Growth of calluses derived from shoot apical meristems of adult and juvenile English ivy (*Hedera helix* L.). Plant Sci. Lett. 22, 387-393.

PREECE J.E. 1987 Treatment of the stock plant with plant growth regulators to improve propagation success. HortScience 22, 754-759.

PREECE J.E. 1997 Use of axillary shoot proliferation in biotechnology. pp. 35-43 *in* Geneve, R.L, Preece J.E. and Merkle S.A. (eds.) *Biotechnology of Ornamental Plants*. CAB International, Wallingord, UK.

PREECE J.E. 2003 A century of progress with vegetative plant propagation. HortScience 38, 1015-1025.

PREECE J.E. & READ P. E. 2003 Novel methods in micropropagation. Acta Hortic. 616, 71-76.

PREECE J.E. & TRIGIANO R.N. 2001 Tissue culture of woody plants pp. 451-456 *in* Jones R.K. and Benson D.M. (eds.) *Diseases of woody ornamentals and trees in nurseries*. APS Press, St. Paul, MN.

PREECE J.E. & IMEL M.R. 1991 Plant regeneration from leaf explants of *Rhododendron* 'P.J.M. Hybrids'. Sci. Hortic. 48, 159-170.

PREECE J.E., HUETTEMAN C.A., ASHBY W.C. & ROTH P.L. 1991 Micro- and cutting propagation of silver maple. I. Results with adult and juvenile propagules. J. Am. Soc. Hortic. Sci. 116, 142-148.

PREECE J.E., VAN SAMBEEK J.W., HENRY P.H. & ZACZEK J. 2002 Forcing the tissue. Am. Nurseryman 196(7), 26-30, 32, 34.

PREECE J.E., IMEL M.R. & SHEVADE A. 1993 Regeneration of *Rhododendron* PJM group plants from leaf explants. J. Am. Rhododendron Soc. 47, 68-71.

PREECE J.E., READ P.E. & BRENNER M.L. 1984 The influence of GA_3 and benzyladenine whole plant sprays on branching and tissue culture of tomato. Bull. Plant Growth Reg. 12(4), 4-8.

PUDDEPHAT I.J., ROBINSON H.T., SMITH B.M. & LYNN J. 1999 Influence of stock plant pretreatment on gynogenic embryo induction from flower buds of onion. Plant Cell Tissue Organ Cult. 57, 145-148.

QUOIRIN M., BOXUS PH. & GASPAR TH. 1974 Root initiation and isoperoxidases of stem tip cuttings from mature *Prunus* plants. Physiol. Veg. 12, 165-174.

RAJASEKARAN K. & MULLINS M.G. 1982 Regeneration of grapevines by aseptic methods. Comb. Proc. Int. Plant Prop. Soc. 1981 31, 213-218.

READ P.E. & ECONOMOU A. 1987 Stock plant influence on tissue culture success. Acta Hortic. 212, 111.

READ P.E. 1988 Stock plants influence micropropagation success. Acta Hortic. 226, 41-52.

READ P.E. & YANG G. 1988 Response *in vitro* of explants chemically treated *via* forcing solutions. Comb. Proc. Intl. Plant Prop. Soc. 38, 406-408.

READ P.E. & YANG G. 1991 Plant growth regulator effects on rooting of forced softwood cuttings. Acta Hortic. 300, 197-200.

READ P.E. & PREECE J.E. 2003 Environmental management for optimizing micropropagation Acta Hortic. 616, 49-57.

READ P.E. & YANG Q. 1987 Novel plant growth regulator delivery systems for *in vitro* culture of horticultural plants. Acta Hortic. 212, 55-59.

READ P.E., GARTON S., CARLSON C.J., CONVISER L. & PREECE J.E. 1979 Programming stock plants for tissue culture success. pp. 197-204 *in* Proceedings, Sixth Annual Meeting Plant Growth Regulator Working Group.

READ P.E., GAVINLERTVATANA P., SURIYAJANTRATONG P., GARTON S. & BRENNER M.I. 1978. Stock plants affect tissue culture success. p. 249. in Hughes, K.W., Henke R. and Constantin M.(eds.) *Propagation of Higher Plants through Tissue Culture*. Technical Inf. Center, U.S. Dept. Energy, Springfield, Va. CONF-7804111.

RITCHIE G.A. & LONG A.J. 1986 Field performance of micropropagated Douglas fir. New Zea. J. For. Sci. 16, 343-356.

ROBB S.M. 1957. The culture of excised tissue from bulb scales of *Lilium speciosum*. J. Exp. Bot. 8, 348-352.

ROBBINS W.J. & HERVEY A. 1970 Tissue culture of callus from seedling and adult stages of *Hedera helix*. Am. J. Bot. 57, 452-457.

ROSS S.D., PIESCH R.F. & PORTLACK F.F. 1981 Promotion of cone and seed production in rooted ramets and seedlings of western hemlock by gibberellins and adjunct cultural treatments. Can. J. Forest Res. 1, 90-98.

ROSSI V., DE PAOLI B. & DAL POZZO P. 1991 Propagation of *Pyrus calleryana* Sel. D6 by *in vitro* culture. Acta Hortic. 300, 145-147.

ROWLAND, L.J., OGDEN, E.L., ARORA, R., CHON-CHONG, L., LEHMAN, J.S., LEVI, A. & PANTA, G.R. 1999. Use of blueberry to study genetic control or chilling requirememt and cold hardiness in woody perennials. HortScience.34:1185-1191

SANKARA RAO K. & VENKATESWARA R. 1985 Tissue culture of forest trees: clonal multiplication of *Eucalyptus grandis*. Plant Sci. 40, 51-55.

SCOTT M.A. 1987. Management of hardy nursery stock plants to achieve high yields of quality cuttings. HortScience 22, 738-746.

SHEVADE A. & PREECE J.E. 1993 *In vitro* shoot and floral organogenesis from stamen explants from a *Rhododendron* PJM group clone. Sci. Hortic. 56, 163-170.

SIMMONDS J.A. & NELSON S.D. 1989 Improved micropagation of *Begonia hiemalis* by maintaining donor plants in long- day conditions. HortScience 24, 831-832.

SMAGULA J.M. & LYRENE P.M. 1984 Chapter 15 - Blueberry. pp. 383-401 *in* Ammarito, P.V., Evans D.A., Sharp W.R. and Y. Yamada (eds.) *Handbook of Plant Cell Culture, Vol 3.* Macmillan, New York.

SONDAHL M.R. & SHARP W.R. 1979 Research in *Coffea* spp.and applications of tissue culture methods. pp. 527-584 *in* Sharp W.R., Larsen, P.O., Paddock E.F. & Raghavan V. (eds*.) Plant Cell and Tissue Culture*. Ohio State Univ. Press, Columbus.

SONDAHL M.R., NAKAMURA T. & SHARP W.R. 1985 Propagation of coffee. pp 215-232 *in* Henke R.R., Hughes, K.W., Constantin M.J., Hollaender A.A. & Wilson C.M. (eds.) 1985 *Tissue Culture in Forestry and Agriculture*. Plenum Press, New York, London.

STIMART D.P. & ASCHER P.D. 1981 Developmental responses of *Lilium longiflorum* bulblets to constant or alternating temperatures *in vitro*. J. Am. Soc. Hort. Sci. 106, 450-454.

STOUTEMEYER V.T. & BRITT O.K. 1961 Effect of temperature and grafting on vegetative phases of Algerian ivy. Nature 189, 854-855.

STOUTEMYER V.T. & BRITT O.K. 1965 The behaviour of tissue cultures from English and Algerian ivy in different growth phases. Am. J. Bot. 52, 805-810.

SURIYAJANTRATONG P. 1979 The influence of *Petunia hybrida* stock plants on growth and differentiation of explants *in vitro*. Ph. D. Diss. Univ. Minnesota Diss. Abs. Int. 40/06 p. 2464-B.

TRIPPI V.S. 1963 Studies on ontogeny and senility of plants. III. Changes in the proliferative capacity *in vitro* during ontogeny in *Robinia pseudoacacia* and *Castanea vulgaris* and in adult and juvenile clones of *R. pseudoacacia*. Phyton 20, 153-159.

VAN SAMBEEK J.W. & PREECE J.E. 1999 Forcing environment affects epicormic sprout production from branch segments for vegetative propagation of adult hardwoods. pp. 272-273 *in* Stringer J.W. & Loftis D.L. (eds.). Proceedings, 12th Central Hardwood Forest Conference, USDA Forest Service General Technical Report SRS-24.

VAN SAMBEEK J.W., LAMBUS L.J., KHAN S.B. & PREECE J.E. 1997a *In vitro* establishment of tissues from adult black walnut. pp.78-92 *in* Van Sambeek, J.W. (ed.) *Knowledge for the Future of Black Walnut.* USDA Forest Service General Technical Report NC-191.

VAN SAMBEEK J.W., LAMBUS L.J. & PREECE J.E. 1997b Production of epicormic sprouts on branch segments of adult black walnut for *in vitro* culture. pp. 93-104 in 88th Annual Report of the Northern Nut Growers Association.

VIEITEZ A.M., SANCHEZ M.C., AMO-MARCO J.B. & BALLESTER A. 1994 Forced flushing of branch segments as a method for obtaining reactive explants of mature *Quercus robur* trees for micropropagation. Plant Cell Tissue Organ Cult. 37, 287-295.

WARREN R.C. 1976 The occurrence of microbes among buds of deciduous trees. Eur. J. Forest Pathol. 6, 38-45.

WELANDER M & HUNTRIESER I. 1981 The rooting ability of shoots raised *in vitro* from the apple rootstock A2 in juvenile and in adult growth phase. Physiol. Plant. 53, 301-306

WELANDER T. 1977 *In vitro* organogensis in explants from different cultivars of *Begonia hiemalis*. Physiol. Plant. 41, 142-145.

WELANDER T. 1978 Influence of nitrogen and sucrose in the medium and of irradiance of the stock plants on root formation in *Pelargonium* petioles grown *in vitro*. Physiol. Plant. 43, 136-141.

WRIGHT N.A. & ALDERSON P.G. 1980 The growth of tulip tissues *in vitro*. Acta Hortic. 109, 263-270.

YANG G. & READ P.E. 1992 Pre-forcing treatments influence bud break and shoot elongation in forced woody species. J. Environ. Hort. 10, 101-103.

YANG G. & READ P.E. 1993 *In vitro* culture of Vanhoutte's spirea explants from 'secondary cultures' and dormant stems forced in solutions containing plant growth regulators. Plant Cell Tissue Organ Cult. 33, 25-30.

Chapter 12
Effects of the Physical Environment

1. COMPOSITION OF THE MEDIUM

Much work reported in the literature suggests that media often need to be varied for different plant genera and according to the kind of culture to be undertaken. It certainly can be advantageous to move a culture from one kind of medium to another when the pattern of growth needs to be modified, or morphogenesis induced. However, many different kinds of plant cultures can be grown on one kind of medium and a critical view would be that much work on the effects of media composition is highly empirical and many workers fail to provide adequate justification of why particular additions to the media are made. We can save ourselves much time and expense if we can modify plant production and use media with fewer constituents and/or change media less frequently during plant development. Murashige and Skoog (1962) (MS) medium has proved to be suitable for callus growth, direct and indirect morphogenesis, and shoot culture of many plant species. This is clearly a good starting point for plant production but we should bear it in mind that direct shoot formation can occur on the explants of some species in water alone. A very simple medium containing sucrose and a relatively low concentration of mineral salts is generally all that is required for root cultures and the micropropagation of most orchids, but in some plants both direct and indirect morphogenesis can be very dependent on the medium used for *in vitro* culture, and a particular balance of inorganic, organic, and growth regulator constituents is essential.

Various compositions have been developed to improve the growth of one or more particular tissues in culture. Features of these formulations, which can influence growth and morphogenesis, are discussed in Chapters 3 and 4. However, growth and development of different species and cultivars can be differentially affected by a single medium composition and may be only modified to a small extent by changing the composition of the medium (e.g. in wheat - Lazar *et al.,* 1983; in *Brassica* - Narasimhulu and Chopra, 1988; and in alfalfa - Brown, 1988). Often, shoot regeneration can be more dependent on genotype than on the presence of complex organic additives in the medium.

One of the reasons that so many different recipes have been developed to aid growth and morphogenesis in culture is that different genotypes will accumulate different endogenous concentrations of growth regulators and the sensitivity of different processes to these regulators will also vary with genotype and with the status of other variables. Growth regulator accumulation can be determined but only with some difficulty using expensive physico-chemical technology and/or immunological techniques which themselves need to be validated using physico-chemical techniques such as GC-MS (gas chromatography-mass spectrometry). This means that it is often difficult to do more than investigate empirically the effects of different applied growth regulators on growth and physiology. Certainly technicians should be aware that the influence of particular concentrations of growth regulator will vary with other variables such as photon flux, temperature, inorganic nutrient concentration, pH and the presence or absence of other regulators. For example, Davies *et al.* (2002) show how the influence of ABA on growth and shoot functioning will depend on pH (which may partly be a function of the nitrogen relations of the plant) and calcium concentration local to the site of action in the leaf. For these reasons and others, it is advisable to avoid being too prescriptive with recipes unless all other plant and culture variables are defined. The plant variables will vary dramatically with the age, developmental and physiological state of the mother plant.

1.1. GROWTH REGULATORS

Growth and organogenesis *in vitro* are highly dependent on the interaction between naturally occurring endogenous growth substances (often not defined or only poorly quantified) and the analogous synthetic growth regulators which may be added to the medium. Growth regulators can bring about *de novo* morphogenesis on media containing suboptimal salt concentrations, and considerably enhance morphogenesis on an otherwise optimal medium (Margara, 1969). The most effective growth regulator or combination of regulators will depend upon other medium constituents. Marked differences between the regenerative abilities of plants, apparent on simple media, are likely to be produced by the use of appropriate growth regulators. Cotyledons originating from separate clones of Douglas fir, exhibit clear differences in their capacity to produce

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adventitious buds on a medium without growth regulators; but with the addition of a suitable auxin and a cytokinin, this variation diminishes considerably (Cheng, 1976). This is presumably because the clones differed markedly in endogenous hormone concentration.

1.2. GROWTH REGULATOR GENOTYPE INTERACTIONS

It is often necessary to alter the growth regulator composition and/or concentration for *in vitro* culture according to the species or variety of plant being grown, and so it is wise to test any new genotype on media containing several different combinations of regulators. There are many examples of this genotype-induced specificity in the literature. The following illustrate the types of interactions commonly experienced.

The concentration of 2,4-D required for the development and further growth of callus tissue from *Phaseolus* hypocotyl tissue was shown by Mok and Mok (1977) to depend upon the species and variety of plant from which the explants were taken. The growth regulator was inhibitory to *P. lunatus* genotypes at 0.55 mg/l but 2.2 mg/l was required to arrest callus growth of the *P. vulgaris* varieties examined. Callus of *P. lunatus* cv. 'Kingston' showed near optimum growth at 4.4 mg/l 2,4-D, whereas that of line PI 194314 was inhibited at this concentration. There was also an eight-fold difference in the auxin requirement of 'Romana' and two other *P. vulgaris* genotypes. Mok *et al.* (1982) have reported similar differences between *Phaseolus* genotypes in respect of their response to cytokinins. The activity of thidiazuron was approximately equal to that of zeatin in promoting the growth of callus of *P. lunatus* cv. 'Jackson Wonder', but in line PI 260415 it was at least ten times higher than that of zeatin. 'Jackson Wonder' callus showed cytokinin autonomy after treatment with either substance, whereas line PI 260415 remained cytokinin-dependent under all conditions tested.

Results of this kind suggest that endogenous growth regulators will have a significant influence on the effects of applied regulators. As physico-chemical and immunological techniques for quantification of endogenous regulators become cheaper and easier to use, we can reduce the necessity for testing the effects of endless combinations of different hormones at different concentrations. These techniques are expensive, however, and will not be available in all labs. Therefore central analytical facilities may be advantageous for the industry.

2. CONSISTENCY OF THE MEDIUM

2.1. USING SOLID OR LIQUID MEDIA

2.1.1. Semi-solid media

The rate at which cultures grow and produce shoots during micropropagation can be influenced by the physical nature of the medium. For many purposes it is convenient to make media semi-solid by incorporating a gel such as agar (see Chapter 4). The chief advantages of using a solidified medium are that:

- small explants are easily seen and recovered
- explants retain the same orientation throughout culture
- plant material is held above the medium so that no special means of aerating the culture is used
- shoots and roots grow in a more orderly fashion because the medium is stationary. In a moving liquid medium growth can be disorientated, shoot buds may be initiated but fail to grow into shoots; and in shoot cultures, separation of shoots for micropropagation can be difficult
- in an agitated liquid medium, callus may break up and/or shed cells to form a suspension culture. This does not occur on a solid medium.

There are disadvantages with the use of semi-solid media. Some agars contain inhibitory substances which may prevent morphogenesis in certain cultures (Powell and Uhrig, 1987), rates of growth can be slow (see below), toxic exudates from explants do not diffuse away quickly. One problem with the use of semi-solid media that has not received much attention is that of poor oxygen diffusion to roots developing in the medium. Rather surprisingly, recent work by Verslues *et al.* (1998) suggests that this may be a problem even in aerated liquid media. Low oxygen tensions will affect both root growth and function and is probably one of the reasons that plantlets from culture often show lesions in root functioning after transplanting, with potentially very serious consequences for plant water relations and the quality of plants produced (see Chapter 13).

Gel from solid media adhering to roots may cause problems for propagators when they are transferring plantlets to soil. A further disadvantage is that the use of semi-solid media increases the time taken to clean containers and glassware for re-use. Vessels may need to be autoclaved to re-melt the agar,

scrubbed and hand rinsed before being placed in a dishwasher.

2.1.2. Liquid media

As explained in Chapter 1, liquid media are necessary for suspension cultures but can also be used advantageously for the culture of callus and organs. Their use often results in faster rates of growth than are possible on semi-solid media. This is because a greater surface area of the explant is in contact with a liquid, and, providing the medium is agitated, the diffusion gradients for nutrients and for gases between it and the explant are reduced. These two factors combined, enable a more efficient uptake of nutrients and growth regulators. In addition, toxic metabolites, which may accumulate in the vicinity of the tissue are effectively dispersed and the partial pressure of oxygen in the root tissues will be increased.

Because plant material must be supplied with oxygen, the uses to which a static liquid medium can be put are very limited. Aeration may not be a problem in cultures contained in very small volumes of liquid (e.g. hanging drops), or in those plant parts submersed in shallow layers of liquid medium. Where a static liquid is to be used for the culture of large pieces of tissue or organs, explants must either be placed in a very shallow layer, so that some of the tissue protrudes above the surface; must float upon its surface; or be supported above the medium. A supply of oxygen for submerged cells, tissues or organs in large volumes of liquid media is normally obtained by shaking, rotating the containers, stirring the medium, or by introducing a flow of sterile air (or a controlled mixture of gases) into the culture vessel. These measures also have the effect of suspending and moving the cultured cells or tissues in the medium but as noted above, this treatment may not be adequate to avoid the development of hypoxia in roots. This may result in the accumulation of toxic metabolites, which will disturb root function. Ethylene will be produced in enhanced quantities at low oxygen tension and this metabolite can dramatically restrict shoot growth and function (Sharp, 2002), particularly if the gas accumulates in vessels where seals restrict gas exchange with the outside air (see below). There may also be direct effects of low oxygen tensions on membrane functioning and on water and ion uptake capacity (Jackson *et al.,* 2003).

Anthers float on a liquid medium, but microspore-derived embryos or calluses are more dense and will sink if the dish is disturbed or moved: they may then die from lack of aeration. Small organs or small pieces of tissue can be made to float more effectively on liquid media if its density is increased by the addition of Ficoll. Anther-derived callus of barley (Kao, 1981) and embryos of *Triticum* could be made to float on the surface of a liquid medium, where they received improved aeration, when 100 g/l Ficoll (Type 400) was incorporated in the medium. In wheat, this treatment resulted in a high proportion of embryos developing into plants: growth in a standard liquid medium was less effective (Jones and Petolino, 1988).

Just occasionally a liquid medium is disadvantageous. Maize cells in suspension culture produce a mucilage, which interferes with cell growth: the formation of a very large amount of mucilage similarly hampered the culture of *Phragmites australis* embryogenic callus (Straub *et al.,* 1988). The motion within liquid cultures may damage delicate tissue. The growth of 'meristemoid-like' green globular bodies (produced from *Asplenium nidus* rhizome segments) was unsuccessful on rotated or shaken liquid medium. Higuchi and Amaki (1989) suggested that this might have been due to the meristems on the surface of the globular bodies suffering from collision damage. One serious disadvantage of using liquid media for shoot growth and multiplication is that shoots, which are perpetually submerged in liquid cultures may have a water-soaked appearance (i.e. they are 'hyperhydric' - see below and Chapter 13) and will then be useless for micropropagation.

For certain kinds of culture, the disadvantages of a semi-solid or liquid medium can be overcome by growing cells, tissues or organs on a porous material irrigated with a liquid medium (see Chapters 2 & 4).

A liquid medium can be used successfully for shoot cultures. It is usual at Stage I to establish explants on an agar medium, and afterwards transfer them to a rotated or shaken liquid medium to promote rapid shoot growth at Stage II. This method was used for instance with carnation (Earle and Langhans, 1974a; Takayama and Misawa, 1982), *Colocasia esculenta* (Jackson *et al.,* 1977), *Eucalyptus* (Mascarenhas *et al.,* 1982), chrysanthemum and periwinkle (Takayama and Misawa, 1982). Axillary shoots are separated and rooted on an agar medium at Stage III or else are rooted *extra vitrum*. Jackson *et al.,* (1977) reported that *Colocasia* shoot tip or axillary bud explants grew well when placed on

liquid medium at Stage I. There was no lag period as on agar medium.

2.1.3. Differential effects

Miller and Murashige (1976) demonstrated that selection between agar-gelled and liquid media (whether shaken or stationary) should not be undertaken arbitrarily. Shoot cultures of four kinds of tropical foliage plants responded differently to liquid or solid media during explant initiation (Stage I) and shoot multiplication (Stage II).

In these experiments, *Cordyline* explants at Stage I survived equally well on solid or liquid medium, but the latter was preferred because it induced greater shoot elongation. None of the *Scindapsus* explants stayed alive on a rotated liquid medium, only a small proportion survived on a static liquid medium, but nearly all grew satisfactorily on a filter paper support or on an agar medium (the solidified medium giving the better shoot growth). The fastest rate of *Dracaena* shoot multiplication at Stage II was obtained from explants grown on filter paper supports at Stage I. Culture of *Cordyline* or *Scindapsus* on static liquid medium at Stage II caused shoots to elongate rather than proliferate.

Wimber (1965) showed the advantage of using a liquid medium for the micropropagation of *Cymbidium* orchids. On a solid medium there was only a small amount of protocorm multiplication and protocorms tended to differentiate into shoots. On a shaken liquid medium, protocorms of most clones continued proliferation. A rapid rate of micropropagation was achieved by transferring protocorms produced in shake culture back to an agar-solidified medium, where, left undisturbed, they gave rise to plantlets. Similar results were obtained on *Cattleya* and *Dendrobium* orchids by Scully (1967) and Sagawa and Shoji (1967). Liquid medium has since often been found to enhance morphogenesis or the rate of shoot multiplication in other kinds of plant.

Single shoots of *Stellaria* did not form multiple shoots on a static medium, but gave rise to a mass of proliferating shoots in moving liquid culture (Walkey and Cooper, 1976). The leaves and stems of cocoa shoot tips elongated on liquid medium, but on agar, growth was restricted to bud swelling. Orchard *et al.* (1979) thought this was because on agar the cut surfaces of the explant became covered with a viscous exudate, which apparently restricted the uptake of nutrients. There was a higher frequency of adventitious leaf (shoot) regeneration from *Arabidopsis* callus on liquid than on agar-solidified medium (Negrutiu and Jacobs, 1978) and in experiments of Grewal *et al.* (1979), callus of *Hyoscyamus muticus* produced some complete plantlets on liquid medium, whereas on a solid medium the callus only proliferated. Callus of *Tylophora indica* formed somatic embryos in liquid medium, but they did not develop further unless the callus was transferred to a supplemented agar medium (Rao and Narayanaswami, 1972). When zygotic embryos of cocoa were grown in liquid culture, their development was more typical of that *in vivo*. Compared to embryos cultured on a semi-solid medium, there was additionally a higher incidence of asexual embryogenesis (Pence *et al.,* 1980).

Results of the kind detailed above suggest that if propagators are to avoid having to develop bespoke micropropagation schedules for individual species and even for different cultivars then we need a better understanding of developmental plant physiology, particularly as influenced by physical structure and the gaseous composition of the medium of plantlet growth. The development of cellular and molecular techniques will allow a greater understanding of the physiological lesions that may develop in plants at low oxygen tensions and will also provide convenient cellular and molecular markers for easy identification of culture-induced lesions.

2.1.4. Propagation rates

Shoot cultures of several different kinds of plants can be multiplied satisfactorily *in vitro* at Stages II and III, when partly submerged in shallow layers of liquid medium without any agitation. Some micropropagation laboratories favour this technique for shoot cultures as it avoids the addition of expensive agar to the medium. Plantlets are also finally obtained without having to wash agar from their roots. Davis *et al.* (1977) were able to grow Stage II carnation shoot cultures in 50 ml liquid medium in 1000 ml flasks (i.e. a shallow layer) with only gentle agitation (one horizontal revolution per minute). Hussey and Stacey (1981) grew single nodes of potato in liquid culture within Petri dishes. The shoots, which were produced, developed roots naturally and so there was no need for a separate rooting stage.

2.1.5. Mass propagation using liquid media

Most attempts at producing very high numbers of plants by *in vitro* methods rely upon the use of liquid media for shoot or shoot meristem multiplication (see Chapter 2 & 4). Attempts to scale-up shoot culture in very large vessels of liquid medium have in the past

been largely frustrated by the problem of hyperhydricity (see Chapter 13) but recently significant progress has been reported (Hvoslef-Eide & Preil, 2005).

The differential results obtained on liquid and solid media can often be effectively combined to obtain the most efficient micropropagation system, e.g. shoot initials formed on an agar-solidified medium can be transferred to a liquid medium to produce rapid shoot growth. Simmonds and Cumming (1976) found that callus of *Lilium* hybrids could be multiplied most rapidly on a liquid medium, but that for the maximum rate of plantlet production it was necessary to transfer the tissue to solid medium. A very similar method of propagating daylilies was described by Krikorian and Kann (1979).

Although bulblets multiplied in liquid culture can be normal phenotypically, the multiplication of leafy shoots submerged in a liquid culture medium can be more problematical. A high rate of multiplication may occur, but as mentioned above, shoots frequently become hyperhydric. This was experienced, for example, with chrysanthemum (Earle and Langhans (1974b), carnation (Earle and Langhans, 1975; Davis *et al.,* 1977), peach (Hammerschlag, 1982) and *Alstroemeria* (Pierik *et al.,* 1988b). When removed from the flasks, shoots were water-soaked and brittle. The leaves of hyperhydric shoots are often abnormally broad and thick, and may not show normal cuticular development (see Chapter 13). Shoots of this kind are easily damaged by desiccation or excessive sunlight and survive very poorly when subcultured or transferred to the external environment. Even without obvious hyperhydricity, shoots of micropropagated plants can show lesions in growth, development and function, which can adversely affect survival after transfer to the soil (Santamaria *et al.,* 2000). These symptoms can often be attributed to low oxygen tensions in propagation media, but accumulation of other gases in the culture vessels can also be a problem (Davies and Santamaria, 2000) (and see below).

2.1.6. Double-phase media

Several workers have experimented with adding a layer of static liquid medium to the top of a semi-solid medium. Johansson *et al.* (1982) found that somatic embryogenesis could be induced more effectively from anthers if they were cultured upon a liquid medium over a layer of agar-solidified medium containing activated charcoal. Maene and Debergh (1985) tried a two-layered medium as a possible method of minimising the cost of elongating and rooting shoots from cultures of ornamental plants. The technique was effective on *Begonia tuberhybrida* shoot clumps. Shoot elongation could either be brought about by moving the clumps from the initial medium to an agar-solidified elongation medium or, a liquid elongation medium could be poured onto the surface of the initial agar medium without transferring the cultures (Viseur and Lievens, 1987).

The combined use of both solid and liquid medium has been patented in Hungary as an improved method of *in vitro* mass propagation (Molnar, 1987). In his paper Molnar says that shoot cultures of a wide range of plants including dicotyledons, monocotyledons and ferns, have been found to produce more axillary buds and shoots in a 2-phase system than on a comparable semi-solid medium. The micro-cuttings obtained were also more vigorous and formed strong roots more rapidly. The best results were obtained when both auxin and cytokinin growth regulators were put into the solid phase, but only cytokinins into the upper liquid medium. Explants seemed to take up nutrients and growth regulators from both the lower and upper layers: presence of the basal solid medium ensured that explants were suitably anchored.

Viseur (1987) discovered that while *Pyrus communis* shoot tips produced a high number of axillary shoots on liquid medium, the shoots soon became hyperhydric and unsuitable for propagation. There was no hyperhydricity on a medium solidified with 5-8 g/l agar, but the number of axillary shoots per explant was then low. The rate of shoot multiplication could be increased however, by adding a layer of liquid medium above the agar medium. Varieties very prone to hyperhydricity required 8 g/l agar in the solid phase: otherwise the best rate of shoot multiplication occurred with 5 g/l. A culture medium had to be present in both the solid and the liquid phase. The use of water-agar or water in the liquid phase, led to hyperhydricity and a decrease in both shoot quality and yield. Molnar (*loc. cit*) emphasised that the upper layer of liquid medium must coat the explants, while Viseur (*loc. cit.*) says that the best results are obtained when the tops of shoots are not quite covered with liquid.

The suitability of 2-phase shoot culture for pear has been confirmed by Rodrguez *et al.* (1991). Chauvin and Salesses (1988) have reported that the use of a double phase medium improved both the

number and the length of axillary shoots in shoot cultures of *Castanea sativa* and *C. crenata*.

Others have found that nutrients can be replenished by adding a layer of liquid medium above a semi-solid medium once it has become exhausted. Shoot cultures which would otherwise need to be sub-cultured can be maintained in this way, thereby reducing labour costs and the stress which is imposed on explants during subculture (Aitken-Christie and Jones,1987; Vermeer and Evers, 1987).

The use of two phase media to minimise the necessity for transferring explants to new media in new vessels must be a potential cost saving development and presumably will also reduce plant losses due to physical damage. This apart, it is difficult to understand the physiological justification of placing different hormones in different compartments (liquid versus solid) and precisely adjusting the depth of the liquid media for different cultivars. There must be a good case for an increased research effort to simplify this aspect of the micropropagation process.

3. THE GASEOUS ENVIRONMENT

3.1. OXYGEN TENSION AND REDOX POTENTIAL

3.1.1. Control of oxygen supply

Although a small flow of gas into and out of cultured plant tissues will be caused by fluctuations in the temperature and atmospheric pressure of the growth room, most of the exchange of oxygen and other gases, is due to diffusion (Jackson *et al.,* 1987). The available oxygen within cultured plant tissues is therefore influenced by:

- The concentration of the gas in the ambient atmosphere
- its rate of diffusion into the culture vessel; and
- its rate of diffusion into the cultured cells or tissues.

The concentration will be closest to that in the ambient atmosphere when the tissue stands free of the medium and is surrounded by a minimum film of moisture or medium. Submerged tissues or organs in a static medium are very poorly aerated.

3.1.2. The ambient atmosphere

The simplest way to influence the oxygen supplied to tissue cultures is to alter the concentration in the external atmosphere. The concentration of oxygen in air is normally close to 21% by volume. In a chamber, oxygen can be maintained at a concentration, which is above or below that in ambient air. The partial pressure of oxygen in the chamber air, is only a general guide to the amount of oxygen available to cultured tissues. An experiment on the effect of oxygen on cultures is most useful if, besides pO_2, the permeability of the culture vessel to oxygen is also described (see e.g. Jackson *et al.,* 1991). Greater accuracy can be obtained by measuring dissolved oxygen instrumentally at a location, which is either very close to the plant tissues or even inside the plant organs. Instruments that can achieve this end are now readily available (Ober and Sharp, 2003) and are providing some surprising results suggesting that most plants grown in aerated nutrient solutions with unrestricted oxygen supply to shoots may also show some limitations in root function due to limited oxygen supply to the roots.

3.1.3. Gaseous environment in the container

If sterilised air, or a gas mixture, is introduced directly into the culture vessel, problems of gas diffusion from the growth room air clearly do not exist. However with unventilated cultures growing in sealed vessels, oxygen concentration at the level of the medium or the tissues can be considerably less than that found externally. This is because use of oxygen by the culture creates a local deficit, which may not be immediately compensated because of the impedance to diffusion created by closures, especially if they are tightly fitting and impermeable. Kozai *et al.* (1988) found that there were 0.1 air changes per hour inside 47 ml tubes if they were covered with aluminium caps, 1.0 change when they were plugged with formed plastic caps, but 6.2 changes per hour if the tubes were covered with microporous polypropylene.

The shape of the culture vessel will influence gaseous diffusion. Not surprisingly, Bateson *et al.* (1987) have shown that in vessels of equal volume, growth is rapid where the diffusion distance is small, but is diminished as the length of the container is increased relative to its diameter.

The concentration of oxygen within the tissues of cultures grown predominantly above the surface of the medium is largely influenced by the partial pressure of the gas within the vessel. The leaves of shoot cultures can therefore absorb oxygen from the surrounding atmosphere as they would *in vivo* (unless they are covered with a water film). Meristem tips on a filter paper supported above a liquid medium are

probably better aerated than if they were placed on an agar medium.

One gaseous component of the atmosphere that has received little attention is the carbon dioxide concentration. It would seem clear that an elevated CO_2 concentration should enhance growth of plantlets that are autotrophic, but this may not always the case, since high sugar concen-trations in the medium can suppress CO_2 fixation (Van Huylenbroeck *et al.,* 1998). Some plants have stomata that are very sensitive to high CO_2 levels and rather counter-intuitively, this can result in low CO_2 concentrations in the leaf, even when the vessel concentrations are high. This is sometimes the case (Santamaria *et al.,* 2000) although in many situations the CO_2 within vessels is reduced by some plant fixation. Here also, low CO_2 will increase the susceptibility of the plant to oxidative stress if the incident radiation becomes too high. Such stress can severely limit plant growth and function.

3.1.4. Diffusion into the medium

Oxygen is only sparingly soluble in water. At 0°C, a solution oxygenated to saturation point from air contains only 10.2 cc oxygen per 1000 cc, and the amount which can be dissolved decreases as the temperature rises. Thus, increasing the incubation temperature of a culture from 21°C to 25°C will decrease the maximum amount of oxygen that can be dissolved in the medium by approximately 9%. Because air is a mixture of gases, more oxygen can be dissolved if water, or an aqueous solution of compounds, is flushed with pure oxygen gas at barometric pressure.

Slightly less oxygen can be dissolved in a plant culture medium than in water because of a phenomenon called the 'salting out effect'. Dissolved salts and non-electrolytes such as sucrose, diminish the solubility of gases. In addition, the concentration of oxygen in plant culture media will usually not reach the saturation level. That actually found will depend on the surface to volume ratio of the medium in the vessel and the concentration (partial pressure) in the immediate gas phase. The former will be smaller when an identical volume of medium is placed in a test tube rather than a Petri dish. Surface to volume ratio is increased when liquid media are agitated or when gaseous mixtures are injected into them. The rate of oxygen uptake from air then depends on the rate of agitation or the rate of gas input into the medium. In a 30 ml flask it was found to be 700 ml/hr at a shaking rate of 100 cycles/min and 1500 ml/hr at 140 cycles/min (Long, 1961).

Oxygen diffuses considerably more slowly through water than through air (respective coefficients of diffusion at 25°C, 2.4 x 10^{-5} cm^2/s and 0.205 cm^2/s: Jackson *et al.,* 1987). Unless a medium is agitated, oxygen will only be supplied very slowly to tissues submerged at any depth. The oxygen uptake of tissues on semi-solid medium is therefore predominantly through those parts which are exposed above the surface.

The passage of oxygen into plant tissues from a gas phase does not seem to present much of a restraint to normal Krebs' cycle respiration, although steles of plant roots have been shown to have very low partial pressures of oxygen.

Soffer and Burger (1988) have obtained evidence that diffusion of oxygen from stationary water into plant tissues is impeded at the water/tissue interface. This resistance to diffusion is diminished but not eliminated if the water, or medium, is stirred or agitated (see also Ober and Sharp, 2003). Even a film of liquid over an otherwise exposed explant will reduce the rate of oxygen uptake into the tissues: the thicker the covering, the greater will be the barrier to the passage of the gas (Konings and Jackson, 1979).

3.1.5. Oxygen requirements of cultures

Partial pressures of oxygen in plants undergoing tissue culture are not often described in scientific papers. There is some evidence that the lowest tolerable level may vary according to plant species (Crawford and Braendle, 1996) but all plants require oxygen to survive, grow and develop. Much of the differential behaviour of plant cultures on liquid and solid media described in the literature may be related to differences in oxygen availability.

Respiration. Plant growth and functioning requires energy, much of which can be provided from metabolism of carbohydrates. The most common and efficient method of energy production is via the Krebs' cycle. This takes place in both light and dark, although it is often called 'dark' respiration to distinguish it from photorespiration. Krebs' cycle respiration requires oxygen: its rate usually decreases immediately the concentration of oxygen falls below that in air. Anaerobic respiration will provide energy for growth and functioning but the energy released from carbohydrate metabolism in this way is much reduced (e.g. Fitter and Hay, 2001).

The growth of potato callus can be doubled if the tissue is moved from air to an atmosphere containing

70% oxygen. Van der Plas and Wagner (1986) showed that this was because respiration was not at a maximum when the cultures were maintained in air. They suggested that oxygen supplementation could be generally employed to increase the rate of growth of callus cultures without inducing injury.

Growth. The rate of plant growth can be diminished by limiting oxygen supply (hypoxia, hypooxia). It can be seen to slow down at significantly higher oxygen partial pressures than those that markedly restrict respiration.

In a natural environment, roots frequently have to grow in low oxygen tensions: root cultures have been said to tolerate relatively low levels of oxygen availability (Street, 1957, 1969). However, the growth of roots in hydroponic solutions is increased by aeration, and cultured tomato roots will also grow more rapidly if a liquid medium is subjected to a continuous gentle agitation (Said and Murashige, 1979). Roots initiated on the shoots of some plants can be seen to grow on the surface of agar, rather than down into it, suggesting that oxygenation of the medium is limiting. The rate of growth of plant roots grown on media solidified with 1% agar can be almost doubled if the plants are placed instead onto 3.5% nutrient agar, which has been crumbled to make it porous (Barrett-Lennard and Dracup, 1988).

The rate of elongation of maize roots in a humid atmosphere, is reduced to only 50-60% of the maximum, when the partial pressure of oxygen falls to 0.1 atm (10 kPa, 75.2 mm Hg), or in a liquid medium, to ca 0.3 atm. Saglio *et al.* (1984) attributed the reduction in growth to the effect of oxygen on non-respiratory processes with a low affinity for oxygen. They found that cell division (the number of cells produced or the number of mitotic divisions) was not affected until the partial pressure of oxygen in the gaseous environment was reduced to 1 kPa (0.01 atm). The formation and growth of adventitious roots on microcuttings is also oxygen-dependent.

The rate of uptake of sucrose, nitrate and other nutrient ions by roots or unorganised tissues is reduced in conditions of hypoxia, although nitrogen supply may not limit growth even when the oxygen in solution is as low as 0.003 $mmol^{-1}$, for some uptake still occurs (Kessel and Carr, 1972; Buwalda and Greenway, 1989). Cells die when oxygen levels fall below a critical level (anoxia) and although tissues can survive for extended periods at low oxygen tensions, there is no evidence that plant tissues can grow under these conditions.

Growth of non-photosynthetic tissues *in vitro* can be slowed considerably, apparently without damage, if the oxygen tension of the medium is appreciably reduced, either by supplying a controlled gaseous mixture to the culture or by preventing the diffusion of air by applying mineral oil or silicone overlays. Growth inhibition in these circumstances is due partly to a lack of oxygen, and partly to an accumulation of carbon dioxide in the tissue.

3.1.6. Cultures needing good aeration

Adequate gaseous exchange is essential to ensure a fast rate of multiplication in most tissue cultures, but a satisfactory rate of growth can usually be obtained when the dissolved oxygen concentration is below the aerobic saturation point. The best cell doubling time in suspension cultures of *Euphorbia pulcherrima* in one and two litre flasks, was achieved when the partial pressure of oxygen, pO_2 was 60% (0.12 atm) (Preil *et al.,* 1988). Similarly the best relative growth rate of embryogenic alfalfa suspensions seemed to occur when the medium was aerated so that it was 70% saturated with dissolved oxygen (pO_2 0.14 atm) (Stuart *et al.,* 1987). The rate of growth of the *Euphorbia* culture (above) declined very rapidly if pO_2 dropped below 10% (pO_2 0.02 atm).

A relatively high level of dissolved oxygen appears to be necessary for the formation of adventitious bulblets *in vitro*. Only a very small number of bulblets was formed from *Lilium speciosum* bulb scales in 100 ml of static liquid medium contained in 300 ml flasks (Takayama and Misawa, 1983). However, the number and dry weight of bulblets produced in 50 ml of the same medium unshaken in 90 x 20 mm Petri dishes were high, and the rate of proliferation even slightly exceeded that obtained when liquid medium in the 300 ml flasks was shaken at 180 rpm. Poor aeration in a column bio-reactor reduced the rate of bulblet proliferation (Takayama and Misawa, 1981). By contrast, although shoot growth of potatoes in large fermentors was found by Akita and Takayama (1988) to require conditions of high aeration [(2 litres of medium in an 8 litre bio-reactor supplied with air at 0.1 vvm (vessel volumes per minute)], once shoots of an adequate length had been obtained, they could be induced to produce miniature tubers in a larger volume of medium (6 litres in an 8 litre bio-reactor) where the aeration was less good. Tubers were not formed if shoots were cultured in the larger volume of medium from the start.

3.1.7. *Beneficial effects of a diminished oxygen tension*

There are several reports, which indicate that a reduction in the partial pressure of oxygen can be used to regulate growth and development in plant tissue cultures.

Photorespiration. Although the growth of non-photosynthetic tissue is decreased when oxygen is limiting, that of green shoots or plants in the light may be increased. Intact plants with the C3 method of photosynthesis (most plants) increase in mass about twice as fast as those grown in air when grown in 2-5% oxygen. This is because in such plants as much as 50% of carbon fixed by photosynthesis in normal air is lost through the process of photorespiration. This is a rapid form of respiration specifically associated with substrates produced during photosynthesis through the oxygenase activity of the enzyme ribulose bisphosphate carboxylase/oxygenase (RUBISCO). This enzyme has a high affinity for oxygen and activity is inhibited by O_2 concentrations below those in air, and almost completely repressed when the atmospheric oxygen level is about 1-2% (Zelitch, 1978). Krebs' cycle respiration utilising electron transfer from cytochrome oxidase to oxygen can take place at much lower oxygen concentrations. In high light, shoots grown at low oxygen tensions can therefore accumulate dry matter quicker than they can in air, but to achieve the most rapid gain, it may be necessary to supply rather more oxygen during the night to permit Krebs' cycle reactions to proceed efficiently.

Shimada *et al.* (1988) have shown that the net photosynthetic rate of green shoot cultures of *Primula malacoides* and *Chrysanthemum morifolium* increases by about 1.5 times when they are grown in an atmosphere containing 10% oxygen instead of normal air. The net photosynthetic rate in 1% oxygen was about 3 times that in air, and was comparable to that of plants growing *in vivo*: plants were autotrophic and grew using only carbon derived from carbon dioxide, without sugar being added to the medium.

Differentiation. The nature of cell differentiation in culture may be regulated by the available oxygen concentration. Dalton and Street (1976) found chloroplast development in suspension cultures to be favoured by low partial pressures of oxygen and high CO_2 concentrations (5-10%). Ethylene, naturally produced by the cultured cells, was antagonistic. Friable callus strains (which may admit more oxygen or may have insufficient internal thickness for full respiratory inhibition) are less liable to develop chlorophyll than compact calluses from the same plant tissue (Street, 1977b). The effect of oxygen on chloroplast formation may be unusual, because other kinds of development in plant cells and tissues appear to be promoted in oxidative conditions (see below). Lignin synthesis can be inhibited by antioxidants (Siegel and Porto, 1961).

Organogenesis and embryogenesis. If, in the medium used for suspension cultures of carrot cells, the dissolved oxygen concentration was reduced below a critical level of 1.3 mg/l (41 $mmol^{-1}$ or approx. 16% of the saturation level), small 'meristematic' cells were produced which resembled those formed under the influence of high auxin concentrations. These cells differentiated into embryoids, whereas the larger cells produced at higher oxygen concentrations only gave rise to roots (Kessel and Carr, 1972).

Carman (1988) found that wheat somatic embryos, incubated in an atmosphere containing 3.2 $mmol^{-1}$ oxygen (7.8% oxygen at a total pressure of 760 mm and 25°C, which could produce a maximum of ca. 90 $mmol^{-1}$ dissolved oxygen), formed less callus than in ambient air, but the proportion of embryogenic scutellar callus to non-embryogenic embryo axis callus was increased. The embryoids formed in low oxygen had fewer abnormalities, secondary embryos, or precocious germination than normal, and a high proportion of them germinated to give seedlings. Abnormal *in vitro* growth and precocious germination of immature zygotic embryos of barley was prevented by culture in an atmosphere containing less than 9% oxygen (i.e. less than ca. 3.7 $mmol^{-1}$) (Norstog and Klein, 1972).

As pollen microspores confined within an anther are more easily induced to produce sporophytic embryoids than if they are isolated on culture media, Sopory and Maheshwari (1976) suggested that reduced oxygen tension is also necessary for androgenesis. This hypothesis seems to be confirmed by the results of Harada and Imamura (1983). Culturing *Nicotiana tabacum* anthers at reduced atmospheric pressure, in an anaerobic environment, or in a nitrogen atmosphere containing less oxygen than is found in air, increased the proportion of microspores undergoing direct embryogenesis: this resulted in more anthers producing plantlets and a greater number of plantlets per anther. When incubation was carried out at 28°C and 1 bar, the most stimulatory oxygen concentration was 5% (giving ca. 56 $mmol^{-1}$ dissolved oxygen).

Suspension cultured cells of *Selinum candolii* produced 4-5 times more somatic embryos per fresh weight of inoculum if, after they were plated on a medium lacking an auxin, they were covered with a layer of liquid paraffin oil. Mathur (1991) supposed that this was due to the reduced concentration of oxygen in the medium. As a reduction in the partial pressure of oxygen would be expected to reduce the exposure of cells to oxidative conditions, the above findings are in contrast to the reports of Earnshaw and Johnson (1986), which suggest that oxidative conditions are required for embryogenesis. Jullien (1974) also thought that a relatively high oxygen tension was necessary.

Many seeds with a hard seed coat are able to begin germination under anoxic conditions until the seed coat is ruptured, when normal aerobic respiration takes over. Henrich *et al.* (1981) noted that seeds of some orchids would germinate with very little oxygen, submerged at the bottom of 15 ml static liquid medium. The protocorms passed through their early developmental stages as if they had been on the surface of an agar medium. Other workers have found that in general, callus growth and somatic protocorm proliferation in orchids is promoted in liquid media where there is a good supply of air, while shoot growth is favoured on static liquid media (Alpi and Garibaldi, 1969; Cheng and Chua, 1982).

The oxygen tension of a medium may be one factor determining organogenesis and subsequent bud growth. Proliferation of *Spiraea* shoots was increased (apparently by more adventitious shoots being formed) after shoot cultures were subjected to a period of anoxia by flushing with nitrogen gas (Norton, 1988; Norton and Norton, 1988a).

An unshaken liquid medium was better than a shaken one (or agar) for inducing coconut flower meristems to grow into 'shootlets' (Blake and Eeuwens, 1982).

3.1.8. Physiological effects of oxygen availability

Redox potential. The redox potential (reduction potential) (E_h) of an aqueous solution is a measure of its tendency to either gain or lose electrons (i.e. to oxidize or reduce newly available substances). Redox potential is measured in units of electrical potential difference (volts). The more positive the value of E_h becomes, the greater is the proportion of oxidant to reductant in solution. As E_h becomes more negative, the relative proportion of reductants increases. The resistance that a solution shows to a change in its E_h is known as its poise. This is analogous to the buffering of solutions against changes in pH.

The redox potential of a solution is altered by pH (increasing as pH is reduced); temperature; and in the case of a gas, pressure. Standard redox potentials (E_0) are therefore defined at a given pH, pressure and temperature (usually pH7, 25°C and 1 atmosphere, unless otherwise stated) when the concentrations of reductant and oxidant are equal (i.e. when the compound in solution is 50% oxidised or reduced).

The redox potential of a plant culture medium varies according to the concentration of dissolved oxygen and the respiratory activity of the cells or tissues growing in it. Anaerobic bacteria cannot be cultured until there is very little oxygen in the medium and bacteriologists often estimate the oxygen tension of a medium or culture by using redox-sensitive dyes, which are coloured when oxidised and colourless when reduced. The E_h values at which colour changes occur, vary according to the dye chosen and the pH of the solution.

In the first stage of normal respiration, glycolysis (which does not require oxygen), glucose is converted to pyruvic acid. Pyruvate is normally passed to the Krebs' or tri-carboxylic acid (TCA) cycle, which is the most energy-efficient of the respiratory processes and requires oxygen for the oxidation of carbon compounds to carbon dioxide. In the process of being oxidised, substrates donate electrons, which are passed on through a series of carriers of increasingly positive redox potential to oxygen, which is thereby reduced to water.

When oxygen is deficient, so that the TCA cycle cannot operate, plants can obtain some further energy (but with much less efficiency) by the decarboxylation of pyruvate to acetaldehyde and the reduction of this compound to ethanol (fermentation). The presence of these two last compounds in tissue culture vessels indicates that glycolysis has been followed by fermentation in some parts of the cultured tissues (Jackson *et al.,* 1987): both chemicals have been commonly detected where the presence of volatiles has been examined [e.g. in flasks containing callus cultures - Thomas and Murashige (1979a,b); or in *Prunus* shoot cultures - Righetti *et al.* (1988)].

Plants are also capable of respiration in the presence of oxygen by another route, the 'hexose monophosphate shunt' or the 'pentose phosphate pathway'. In tobacco callus this method becomes much more dominant during adventitious shoot formation (Thorpe and Laishley, 1973; Brown and Thorpe, 1980). Although the pentose phosphate

pathway is slightly less energy-efficient than glycolysis linked to the oxidative-TCA cycle, it allows for the formation of higher concentrations of reduced nucleotides and 5-carbon intermediates, important for the synthesis of nucleic acids.

Some workers have suggested that development is associated with hypoxia: others have thought it to be an oxidative process. Publications describing the addition of oxidising or reducing agents to plant tissue cultures, have provided evidence supporting both views. Note that, redox potential will affect the availability of inorganic ions in plant cultures because the solubility of metal ions in water can depend on both pH and E_h. For example, Fe^{2+} becomes more soluble as E_h and pH decrease

The frequent addition of hydrogen peroxide (a strong oxidising agent) has been shown to increase the yield of somatic embryos and bring about a decrease in the concentration of glutathione (Kapik *et al.,* 1986). But on the other hand, Harada and Imamura (1983) were able to increase embryogenesis from pollen within *Nicotiana tabacum* anthers by incorporating the reductants ascorbic acid (0.6-568 mM), mercaptoethanol (1-1000 mM) or dithiothreitol (0.6-648 mM), and Joy *et al.* (1988) found that the addition of 40-80 mM ascorbic acid to a shoot-forming medium, enhanced adventitious shoots in both young and old *Nicotiana tabacum* callus.

The concentration of reduced glutathione, another reducing agent, has been noted to be higher in proliferating callus than in embryogenic callus of wild carrot (Earnshaw and Johnson, 1985), suggesting that oxidative conditions were required for embryogenesis. This result is supported by the later finding that adding reduced glutathione to the growth medium prevents embryogenesis in carrot tissue cultures: the addition of other antioxidants (ascorbic acid and vitamin E), can have a similar effect (Earnshaw and Johnson, 1986). By contrast 10 mg/l glutathione (presumably GSH) has been added to media for the induction of embryogenesis in *Medicago sativa*, apparently without inhibitory effects (Senaratna *et al.,* 1989).

Tissues often become brown and necrotic after excision due to the presence of activated oxygen and other oxidising substances. Several authors have noticed that tissue browning and necrosis is not necessarily detrimental to morphogenesis, and Krul and Worley (1977) found that somatic embryos of *Vitis* all arose adjacent to areas of necrotic cells. In *Paulownia tomentosa*, the presence of necrotic tissue was apparently essential for the initiation of embryogenesis (Radojević, 1979).

Reducing agents. It is sensible to include a discussion of the effects of reducing agents on *in vitro* cultures at this point, even though they are not factors of the external environment.

Ascorbic acid. L-ascorbic acid (ascorbate, Vitamin C) is synthesised by plants and occurs naturally in cells in relatively high concentrations [e.g. 13 mg/100 g fresh weight of suspensions of *Rosa rugosa* cultured in the dark (and even more in the light) (Wegg and Townsley, 1983); 23.4 g/100 g chlorophyll in protoplasts (Foyer et al., 1983)]. Ascorbate acts as a reductant, being converted by oxidation to dehydro-ascorbic acid. Reconversion of dehydroascorbic acid to ascorbic acid, by the enzyme dehydroascorbate reductase, brings about the oxidation of glutathione.

Ascorbic acid, which is water soluble, and Vitamin E, which is hydrophobic and associated with membranes (see below), provide cells with a defence against oxidative injury. Vitamin C can scavenge injurious hydrogen peroxide (Foyer *et al.,* 1983; Thompson *et al.,* 1987) and some other activated oxygen radicals. It is responsible for reducing the oxidised form of Vitamin E.

Ascorbic acid has been added to few published media for plant tissue culture and so it is not an essential ingredient. Several researchers have included small quantities (i.e. 0.1-1.0 mg/l) without evidence of a clearly beneficial effect. Definite responses have, however, been measured in some studies. Bonner and Axtman (1937) reported that adding 50 mg/l (but not 25 or 100 mg/l) to the culture medium increased the growth of isolated *Pisum sativum* roots. The validity of this finding has been confirmed by Liso *et al.* (1988) who found that root growth of *Allium cepa* roots was increased over periods of several weeks by moderate concentrations of ascorbic acid (or its precursor, L-galactono-γ-lactone). It was noted that 0.1 mM ascorbic acid stimulated DNA synthesis in root meristems and appeared to shorten the cell cycle, especially the G1 phase. Chlorophyll formation in callus cultures of several plants was assisted by the presence of 10 mg/l ascorbic acid or ascorbate (Fukami and Hildebrandt, 1967).

Thiourea (thiocarbamide). The reducing agent, thiourea is routinely incorporated into media by some workers (Pua *et al.,* 1983). It has been reported to enhance the growth of *Lactuca sativa* and *Cichorium endiva* callus (Fukami and Hildebrandt, 1967) and to promote the growth of uniform cell suspensions

(Nitsch *et al.,* 1970). Its antioxidant effect has been noted to produce a growth regulatory effect *in vivo*; dormant peach buds, which can be stimulated to grow in partially anaerobic conditions (Erez *et al.,* 1980), can also be forced into growth by sprays of thiourea.

Together with other thiocarbamides, the compound has three notable biological properties: it complexes with metal ions, particularly with those of copper and Fe(III); together with other carbamides, it is (like ascorbic acid) an effective scavenger for hydrogen peroxide (being readily oxidised to a disulphide); and is able to inactivate peroxidase enzymes (Skellern, 1989).

Glutathione. Fuchigami and Nee (1987) have suggested that glutathione has a role in overcoming rest or dormancy in plants. Reduced glutathione is thought to maintain protein thiols in a reduced state, thereby inducing polysome (the association of one m-RNA molecule with several ribosomes) activation and protein synthesis. Like pentose phosphate respiration, high levels of reduced glutathione ensure an above-normal availability of NADPH. It has been proposed that the balance of reduced glutathione (GSH) to oxidised glutathione (GSSH) influences cell extension, growth and cell division (Standardi and Micheli, 1988). The addition of 2 mM glutathione to Gamborg *et al.* (1968) B5 medium, promoted cell division of *Brassica* callus (Sethi *et al.,* 1988). As with embryogenesis, indirect adventitious shoot formation in *Malus* was prevented by 100 mg/l GSH (James *et al.,* 1984).

4. GROWTH REGULATORS

Auxins have been found to activate enzymes regulating respiration via the pentose phosphate pathway, while enzymes concerned with glycolytic respiration are reduced in activity (Black and Humphreys, 1962). Kinetin too has been thought to have the same effect (MacLeod, 1968) and carrot cells prompted to divide by kinetin were noted to consume less oxygen than when this cytokinin was absent (Neumann, 1968). Some cytokinins are capable of acting as reducing agents in photochemical reactions and Rothwell and Wright (1967) proposed that this might account for part of their biological activity.

High levels of the naturally-occurring auxin, IAA, promote active cell division. Enzymes in oxidative conditions readily denature this phytohormone, but breakdown can be prevented by anti-oxidants of various kinds (Stonier *et al.,* 1970). The synthetic auxin 2,4-D, which is frequently used to control the continuation of proliferative growth or the onset of embryogenesis, has been said to elevate cellular antioxidant (GSH) concentrations (Earnshaw and Johnson, 1986), while the addition of ascorbate to culture media decreases it in favour of oxidised glutathione (GSSH) (Standardi and Micheli, 1988). This suggests that it may be important to correlate redox potential with stages of morphogenesis.

For detailed information on plant growth regulators see Chapters, 5, 6 & 7.

5. CARBON DIOXIDE

5.1. GROWTH REGULATION

5.1.1. Non-autotrophic requirement

Low concentrations of carbon dioxide can stimulate ethylene biosynthesis: at higher concentrations (1-10% CO_2) it competitively inhibits ethylene action. It seems likely that many of the affects of CO_2 on tissue cultures may be exerting their influence through a modification of the ethylene effect but there has been some discussion of a non-photosynthetic requirement for carbon dioxide in plant tissue cultures. The presence of carbon dioxide (optimum partial pressure 0.1 atm) may aid initiation of new growth in low-density cultures of sycamore cells in suspensions, or on agar plates. The gas had a conditioning effect: non-photosynthetic fixation resulted in the cells producing organic acids and amino acids, which were essential for growth. Attempts to replace these naturally produced compounds with synthetic alternatives were unsuccessful (Stuart and Street, 1971: Gathercole *et al.,* 1976). These results have been confirmed in other plants. Besides leading to the biosynthesis of organic and amino acids, non-autotrophic fixation has also been found to result in the production of lipids (Nesius and Fletcher, 1975; Obata-Sasamoto *et al.,* 1984; Plumb-Dhindsa *et al.,* 1979; Vanderhoven and Zyrd, 1978).

Concentrations of CO_2 of the order of 2000 ppm can occur in tissue culture vessels (Santamaria *et al.,* 2000) and in many plants, this concentration has been shown to promote bleaching of leaves, particularly when combined with high photon flux densities. This can substantially limit CO_2 uptake

and may be the result of photo-oxidative damage (Santamaria *et al.,* 2000).

5.1.2. Effect on organogenesis

Shoot formation from tobacco cotyledons was inhibited if the gas phase contained 1% CO_2 rather than ambient levels during the first 7 days of incubation. A similar effect was produced by 5 mM aminoethoxyvinylglycine, an inhibitor of ethylene biosynthesis, which led Everett (1982) to suggest that carbon dioxide was antagonising the action of ethylene. By contrast, Cornejo-Martin *et al.* (1979) reported that ethylene (5 vpm) combined with 2% CO_2 was more effective than ethylene alone for promoting shoot formation in rice callus in the presence of BA. Similarly Kumar *et al.* (1986) found that the ethylene and the carbon dioxide which built up in sealed containers after inoculation of *Pinus radiata* cotyledons, were responsible for promoting shoot morphogenesis on a medium containing BA.

6. CONTAINER SIZE

Container volume can sometimes affect growth and morphogenesis *in vitro*. These effects are probably due to different concentrations of oxygen, carbon dioxide, ethylene and other volatiles in the air space within the container. Vessels of different size usually differ in the ratio of explant to medium, explant to air, and medium to air: the shape of the vessel can also influence the growth rate of cultures by modifying the rate of gaseous diffusion (Bateson *et al.,* 1987).

There are many examples in the literature of vessel size having an effect on the rate of culture growth or the rate of shoot proliferation. They usually show that growth is not at an optimum in very small containers: sometimes an optimum size of container has been demonstrated. Fresh weight increase, morphogenesis or the rate of axillary shoot proliferation is frequently affected in different ways. Growth of *Saintpaulia* shoots was greater in 120 ml jars than in 60 ml ones (Start and Cumming, 1976) and shoot proliferation in shoot cultures of several woody plants better in 200 ml or 350 ml vessels than in 60 ml tubes (McClelland and Smith, 1990).

6.1. PRACTICAL RECOMMENDATIONS ON VESSEL SIZE

It is impossible to recommend vessel sizes for any particular type of micropropagation procedure without prior experimentation. The best size and shape of vessel will vary from one species to another, and will depend on factors such as the material from which the vessel is made (e.g. glass or gas-permeable plastic), the type of closures used, the volume of medium and the density of inoculation (e.g. Jackson *et al.,* 1991).

Takayama and Misawa (1981) and, since them, many others (see Hvoslef-Eide and Preil, 2005) have attempted to scale-up the propagation of various kinds of ornamental plants using liquid culture in large vessels. Leaf segments of *Begonia hiemalis* undergoing direct shoot regeneration cultured in jar or bubble column fermentors had a lower growth rate than those in shaken 150 ml Erlenmeyer flasks. This was thought to be due to the relative rates of aeration (Takayama and Misawa, 1981). In general terms, large vessels are labour-saving devices and by using them methods of culture can be simplified permitting the growth of larger plants in less physiologically-demanding conditions.

7. THE PHYSICAL ENVIRONMENT

7.1. TEMPERATURE

In their natural environment, plants usually experience temperatures which fluctuate widely, especially between day and night. It is probably not necessary to mimic this day to night variation but we should be aware that day temperature and night temperature do not always exert the same effects on plant growth (e.g. Langton and Cockshull, 1997). The growth of plant cultures may be improved by lowering the temperature at night because dark respiration will be reduced and where growth rooms have to be heated, there is a saving in fuel costs by reducing the night temperature. Direct effects of night temperature on growth can be more varied, with increasing night temperature decreasing internode length in some species and increasing it in others. A further advantage of alternating temperatures is that they assist the exchange of gases in culture vessels (Chalupa, 1987). However, such variation is not essential and, in many laboratories, tissue cultures are maintained in growth rooms at the same temperature by night and day.

To speed growth and morphogenesis *in vitro*, cultures are generally maintained at mean temperatures which are higher than those which would be experienced by the same plants growing *in*

vivo. The average constant growth room temperature employed in a large sample of experimental reports was found to be 25°C (with a range between 17 and 32°C). Tropical and subtropical plants such as cotton, rice, *Citrus* and *Bougainvillea* tend to be cultured at slightly higher temperatures than temperate species (average 27.7°C, range 24-32°C). Where a diurnal fluctuation is preferred, a somewhat higher-than-average day temperature is usually coupled with a dark-period value that is ca. 4-8°C lower than the daytime figure: typical variations adopted are 25°C day: 20°C night, or 28°C/24°C.

There is not a single defined temperature for the tissue culture of any one species and that employed in published reports will often be found to vary according to the experimenter and the kind of organ or tissue being cultured. Such differences usually have only minor effects on the rate growth or propagation.

Many small micropropagation laboratories have only a single growth room in which temperature is fairly uniform. Different kinds of plant can be successful multiplied under such conditions, but there are frequent examples in the literature to prove that different plant species can have distinct temperature optima. *Dicentra spectabilis* shoot cultures proliferate most rapidly at 22°C and explant growth and shoot proliferation are both poor at 27°C.

Note that in lighted rooms, the temperature inside culture vessels is usually several degrees higher than room temperature, due to the greenhouse effect (Debergh, 1988). In addition, the base of the vessels is frequently warmer than the top.

The following discussion shows how explant establishment, culture growth, plantlet development, and morphogenesis can be temperature-dependent.

7.1.1. Culture establishment and growth

The rate at which plant material grows *in vitro*, usually declines slowly as temperatures are reduced below the optimum, while above the optimum, the rate of growth falls off more rapidly, and cultures also frequently appear to be unhealthy. Although shoot cultures of *Artocarpus heterophyllus* (Jackfruit) produced more axillary shoots at 25 or 30°C than at 20°C, hardly any were obtained at 35°C. Rahman and Blake (1988) supposed that at the higher temperature, respiration exceeded photosynthesis.

Diamantoglou and Mitrakos (1979) discovered a clear temperature optimum for the growth of olive embryos. Although growth was slow initially at 25°C, 70% of the embryos developed into plantlets at this temperature while at 15, 20 or 30°C, the success rate was more than halved. Leaf blade explants of *Vitis* survived in culture at 20 and 25°C, but did not grow. Growth was only satisfactory at 29°C; at 32 and 34°C the explants survived for only a short while (Favre, 1977).

Culturing explanted peach shoot tips at 21-24°C ensured 80-90 per cent survival, but only 7 per cent survived at 28°C (Hammerschlag, 1981, 1982). Callus cultures too, show large differences in growth rate depending on temperature. Tumour callus of sorrel grew rapidly at 21 and 25°C and only poorly at 5, 15 or 30°C. At 36 and 45°C there was little growth and the callus turned brown (Nickell and Burkholder, 1950).

Temperature requirements can sometimes vary between quite closely related genotypes. Having established cultures from stem nodes of 14 different *Rosa* hybrids at 24°C, Horn *et al.* (1988) looked at the effect of a range of temperatures on the rate of shoot multiplication. The best overall results were obtained at 18°C, but certain 'thermonegative' hybrids gave the best results at 12°C while in other 'thermopositive' ones, shoot proliferation was at an optimum at 18 or 24°C.

There are many other examples where the cultures of certain plants require below-average temperatures. For example, the best plantlet growth from the floral meristems in cauliflower curd tissue occurred at 18°C: growth was less at 22°C (Walkey and Woolfitt, 1970). Single node cultures of *Syringa vulgaris* grew satisfactorily at 21°C in a 16 h day under an irradiance of 5 Wm^{-2}, but if the cultures were kept at 25 or 27°C, growth was abnormal and there was leaf yellowing and curling (Pierik *et al.,* 1988a).

7.1.2. Morphogenesis

Shoot bud formation. Organogenesis frequently reaches an optimum within a narrow range of temperatures, which varies between species.

Induction at relatively low temperatures. Adventitious shoot initiation on *Begonia cheimantha* leaf explants has been observed to be at an optimum in leaf cuttings at 15°C (Heide, 1965) and in petiole segments at 15-20°C (Fonnesbech, 1974a). Further shoot development was, however, enhanced at 24°C (Fonnesbech, 1974b). The initiation process is promoted by cytokinins, and in both series of experiments, incubation at 25-27°C inhibited kinetin-induced bud formation. Similarly in *Streptocarpus hybridus*, direct shoot formation on leaf discs (which were not maintained aseptically) was best at an

incubation temperature of 12°C, and fewer buds were formed per disc as the temperature was increased up to 30°C (Appelgren and Heide, 1972). Shoot induction on peduncle explants of *Anemone coronaria* took place only at 15-19°C, and above 26°C most explants died (Sutter and Langhans, 1978).

Induction at high temperatures. In some plants the temperature for the best induction of adventitious organs is somewhat higher than that required for growth. In *Pinus radiata* for example, adventitious buds formed most readily on juvenile explants when they were cultured in a 28°C (day), 24°C (night) regime, whereas the shoots, which were formed grew best at 24/20°C (Aitken *et al.,* 1981). A very different temperature optimum was found for shoot formation for fragmented shoot apices of *Vitis vinifera*. Here the rate of initiation and multiplication of adventitious shoot buds was much greater at 35°C than at 27, although explants were killed at 38°C (Barlass and Skene, 1982).

Induction at normal temperatures. In other plants, shoot regeneration seems to be most effectively induced at temperatures closer to those used for normal tissue culture. On rapeseed (colza) flower stalk segments, where adventitious shoots were readily formed at 24°C, low temperatures (13°C for 16 h day, 6°C at night) inhibited subsequent morphogenesis, especially when applied during the first 1-2 weeks of culture (Margara and Leydecker, 1978). Although in *Brassica campestris*, adventitious shoot formation from leaf discs was better at 20°C than at 25°C, it occurred satisfactorily at several temperatures in *B. napus* and *B. oleracea* (Dunwell, 1981; Johnson and Mitchell, 1978). Very similar results were obtained by Kato and Ozawa (1979) with leaf fragments of *Heloniopsis*. Shoot bud formation (here largely independent of the addition of benzyl adenine) was at an optimum at 21-25°C. Pre-treatment at 30°C for 7 days or more, reduced the number of buds eventually formed in segments of both young etiolated, and mature green leaves, but not in segments of young green leaves unless they were kept in the dark at the same time.

Pretreatments. Morphogenesis is often influenced by the temperature at which mother plants or cultures have been maintained previously. Kato and Ozawa (1979) reported that *Heloniopsis* shoot initiation was enhanced if explants were maintained at 16°C for 7-21 days before culture at a higher temperature and Von Arnold and Eriksson (1979) found that to induce a high proportion of *Picea abies* needles to form adventitious buds, they should be kept at 10-16°C before incubation at 20°C. Even lower temperatures are inductive in other plants (see below). Maintaining tomato stem explants for an unspecified time at a 'low' temperature of 19°C was described as enhancing their regenerative potential (Reynolds *et al.,* 1982).

Selection for a low temperature response. Heating costs involved in the greenhouse culture of ornamentals make it desirable to obtain varieties with an ability to grow and flower in reduced temperatures. Grunewaldt (1988) has reported the selection of clones with an ability to grow at 16°C (instead of the normal 20°C) by low temperature induction of direct shoot regeneration in leaf explants, some of which were from mutagen-treated plants. The earliest clones to flower at 16°C were amongst the plants regenerated by a 7-day incubation at 26°C followed by 12 weeks at 15°C.

Direct adventitious root formation. The formation of adventitious roots on shoots is temperature dependent. For example no callus or roots formed on young shoot tips of *Asparagus* at 0, 10 or 15°C, but 20 per cent of cuttings rooted at 20°C, and 45 per cent at 25°C (Gorter, 1965). In many plants, root induction on the shoot micro-cuttings produced *in vitro* seems to require a slightly lower temperature than is necessary for shoot multiplication and growth.

Conifers seem to root most effectively at a temperature of ca. 20°C (Chalupa, 1987). Shoots derived from Douglas fir shoot cultures grown at 24°C, yielded only a few plantlets if root induction was carried out at this temperature. The plantlets also showed a discontinuity in their anatomy due to the formation of callus at the shoot-root transition zone. On the other hand, many plantlets of normal appearance were produced at 19°C (Cheng, 1978). *Digitalis lanata* shoots from cultures grown at 24°C were best rooted at 19°C/14°C (day/night) (Schner and Reinhard, 1982, 1986) and shoots obtained from *Aconitum carmichaeli* shoot cultures multiplied at 25°C produced more roots at 20°C than at 25°C (Hatano *et al.,* 1988).

Not all plants respond in this way: shoots of some may root better at a higher temperature. Microcuttings of two apple varieties produced at 25°C, rooted well at 24-26°C but rooting was partially suppressed at 20-22°C or 28°C (Kataeva and Butenko, 1987). Zimmerman (1984) obtained similar results with shoots of different apple cultivars from shoot cultures maintained at 25°C. A very high proportion of shoots rooted when placed on a rooting

medium in the dark at 30°C for one week, followed by a 16/8 h regime at 25°C. The rooting of 'Delicious' and 'Vermont Spur Delicious' was improved by incubation at 30°C, while that of 'Royal Red Delicious' was best at 35°C.

Tuber and bulblet production. Incubation temperature is reported to have a marked effect on the rate of *in vitro* tuber formation in potato. At least 10 times more tubers were formed at a constant 20°C than at 28°C, or at a day temperature of 27°C and cooler nights (Wang and Hu, 1982). In bulbous species, culture at certain temperatures can cause the cessation of shoot growth, a type of dormancy, which often requires a cold treatment to reverse it (see below).

Culturing *Lilium longiflorum* bulbscales *in vitro* at 30°C resulted in the formation of bulblets, which produced leaves when transplanted, either without further treatment or after the bulblets had been immersed in water at 45°C for one hour. All the bulblets regenerated at 25°C were fully dormant however, and did not produce leaf axes when transplanted (Stimart and Ascher, 1978; Stimart *et al.,* 1983). Scale sections of *Lilium speciosum* cultured at 20 or 25°C, produced bulblets which were dormant when planted *extra vitrum*; but bulblets produced from section grown at 15°C began growth straight away (Paffen *et al.,* 1990).

7.2. LOW TEMPERATURE TREATMENTS

7.2.1. Callus

The proportion of *Arabidopsis* callus cultures forming shoots and roots was increased when 9 month old callus was kept for 3-6 days at 4°C before transfer to regeneration medium at the normal culture temperature of 25°C. The weight of roots per callus was also increased by this treatment (Negrutiu and Jacobs, 1978).

7.2.2. Cold treatments to reverse dormancy

The value of cold treatments to overcome the natural resting state of buds from which explants are required, is discussed in Chapter 11. A similar cold-sensitive dormancy occurs occasionally during *in vitro* culture, or immediately after plantlets have been transferred to the external environment. It has been most frequently reported in bulbous species where plants often become gradually dormant (particularly if they are not sub-cultured).

Bulbs. Plants which produce a bulb *in vitro* often become dormant; this is sometimes manifested by senescence of the shoot above the bulb (e.g. in onion, Hussey and Falavigna, 1980). Dormant plants or bulbils can often be induced to resume growth or to germinate, either *in vitro* or in an external environment, if they are kept for a few weeks at a low temperature (1-10°C has been found to be appropriate).

Graves *et al.* (1978) administered cold to plantlets of *Lilium*, *Hemerocallis* and *Hippeastrum* at Stage III before they were potted into soil, similarly finding that it reduced the risk of injury and allowed the young plants to initiate rapid growth. Bulblets of *Lilium auratum* and other *Lilium* species produced on MS + 90 g/l sucrose, required 100-140 days at 5°C before dormancy was broken, but those formed on the same medium containing 30 g/l sucrose required only about 50-70 days of cold (Takayama and Misawa, 1980; Takayama *et al.,* 1982).

Where continued subculture is desired, treatments other than cold can sometimes be used to encourage plants to resume active growth. Splitting crowns into two after 12-14 weeks of culture was effective in *Freesia*, but in some other members of the Iridaceae, this treatment resulted in very weak plants (Hussey, 1976). Mahotiere *et al.* (1976a,b) found that onion shoot dormancy could be avoided by transferring cultures from 20°C to 10°C for 96 h. Continued growth could alternatively be promoted by the addition of 100 mg/l kinetin and/or 100 mg/l sucrose to the medium. Dormancy of *Gladiolus*, *Iris*, *Sparaxis* and *Schizostylis* plants *in vitro* was prevented by 0.03 mg/l BA (Hussey, 1976, 1977).

Woody plants. Shoots in cultures of woody plants can become dormant *in vitro*, although this is rare. One reported example is in *Clematis*, where dormancy in many cultivated varieties is triggered by short days and cool temperatures. Shoots derived from shoot cultures of var. 'Elsa Spath' became dormant *in vitro* in short days, especially if this treatment was accompanied by cool temperatures. The dormancy could be broken by 100 mg/l GA_3, or chilling in short days. Placing shoot cultures in long days during the chilling period resulted in the incomplete elimination of dormancy, and subsequent rosette growth (Kratz and Langhans, 1978).

Dormancy in plantlets of woody species is more common when rooted plantlets are transferred *extra vitrum*. They may then fail to grow vigorously or may form an obvious resting terminal bud and cease growth altogether. Dormancy of this kind can usually be overcome by cold, or gibberellic acid, treatments. Plantlets of *Prunus institia* (plum rootstock) remained small with weak growth unless kept for two months at 0°C or given a spray of 200 mg/l GA_3 after potting

(Howard and Oehl, 1981). Gibberellic acid is often added to media used for propagating fruit and nut species to promote shoot elongation (Lane, 1982). Significantly greater growth and clonal uniformity of some *Prunus* and *Malus* species was achieved when plantlets which had been rooted *in vitro*, were kept for at least 42 days at 2-4°C in the dark before they were planted in the greenhouse [Suttle (Driver and Suttle, 1987)].

Dormancy, similar to that occurring in some shoot cultures, may result from embryo culture of species in which seeds require to be subjected to a period of cold moist conditions (stratification) before normal growth. Then embryos dissected out from unchilled seeds may germinate *in vitro* but produce dwarfed plants, typically having a rosette of leaves and virtually no internode growth. Plants may remain in this condition for many years (Flemion, 1959) unless chilled or treated with a gibberellin (Barton, 1956). If kept in constant temperatures, pine callus cultures occasionally cease growing, but will resume active growth at normal temperatures if stored in the cold for about 6 weeks (Bonga, 1977a,b).

7.2.3. The induction of flowering

Cold treatments promote the flowering of certain plants *in vivo*, particularly biennials. The work of Gertsson (1988) has suggested that *in vitro* culture at cool temperatures may have a similar effect. Plants of *Senecio hybridus* obtained from shoot cultures maintained at 10°C, flowered earlier, had a greater foliage height, and taller inflorescences, than those from cultures grown at 21°C.

8. HUMIDITY

Relative humidity (R.H.) is a measure of the amount of water vapour contained in a gaseous atmosphere. It is expressed as the ratio of the quantity of water an atmosphere actually contains, to that which it could contain when saturated.

The R.H. of the gaseous mixture ('air') above the medium within a culture vessel depends on its temperature and that of the medium. Where the temperature of the air equals that of the medium, and the vessel is effectively sealed, the relative humidity should theoretically be in the range 98-99.5%. The air may become almost fully saturated if the temperature of the medium exceeds that of the air, but relative humidity will decrease if there is an exchange of water vapour from the vessel to the growth room.

A relative humidity of ca. 70% is usually recommended for growth rooms. Humidities lower than this can occur in rooms fitted with cooling units, causing media within partly sealed vessels to dry out rapidly. An humidifier should be placed within growth rooms to rectify this situation.

8.1. THE OPTIMUM HUMIDITY FOR CULTURES

Because cultures grown in high humidities are liable to become hyperhydric, it would be ideal to surround shoot and organ cultures with air that is not fully saturated. However, a reduction in humidity will increase the risk of desiccation of tissue cultures. Jones (1974) found that carrot embryoids would not stand desiccation and were best kept at a relative humidity of 80-90%. They all died if humidity was less than 60%. The rate of shoot proliferation of *Pelargonium* shoot cultures was poor when the relative humidity was below 75% (Cassells and Minas, 1983), and cauliflower plantlets exposed to 30% and 15% R.H. *in vitro* experienced high mortality.

The humidity in vessels containing cultured shoots also depends on leaf area development. This is because leaves absorb radiation and commonly exhibit temperatures above the temperature of the air, thereby increasing the driving force for transpiration.

8.2. COOLING THE BASE OF VESSELS

A common situation in growth rooms is for the bases of vessels, resting above a warm light source, to be at a higher temperature than their tops. In these circumstances, if the air in the vessel has a high relative humidity, the top of the vessel may then reach the dew point and water will condense on the sides, or upon the lid, at the coolest point.

The relative humidity inside culture flasks will be less than 100% if the air temperature is higher than the temperature of the medium, for then water in the atmosphere condenses into the medium (Vanderschaeghe and Debergh, 1987). Thus the most effective method of reducing R.H. in culture vessels is to stand them on a cooled platform so that the medium is cooler than the air above it. In high relative humidities, the temperature of the medium then becomes the potential dew point (Debergh, 1986a,b; 1988; Vanderschaeghe and Debergh, 1987; 1988).

In the experiments of Vanderschaeghe and Deberghe (1987), the most cost efficient method of cooling the shelving of growth rooms was found to be

the circulation of a mixture of antigel fluid and water (previously cooled by a cryostat) within metal pipes. Flasks were placed on a metal shelf over the pipes.

8.3. SURVIVAL *EXTRA VITRUM*

Frequently there are high losses when shoots or plantlets which have been grown in enclosed containers under high humidity, are transferred to an external environment. This is partly because they may have become hyperhydric, and partly because they are not adapted to a dry environment. This may be manifest as poor stomatal control of water loss, reduced photosynthetic capacity and poor root growth. The degree of adaptation can be increased by growing shoots in a reduced humidity before transfer, but shoot growth and rooting capacity may decline progressively as humidity is lowered (Short *et al.,* 1987). This means that such treatments are only practical for a short period before transfer. Normally cultures are grown in high humidity until they are removed from the culture vessel and only then are shoots gradually hardened to the harsher external environment.

9. THE EFFECTS OF THE PHOTOENVIRONMENT

Most commonly in micropropagation, the photo-environment is quantified as light intensity, illuminance or irradiance. This is radiant energy of those wavelengths which produce visual sensations to the eye. The visible radiant energy spectrum encompasses wavelengths from about 390 nm (violet), to around 700 nm (red region).

A range of units has been devised to compare the luminous qualities (illuminating capabilities) provided by light sources. These units are not ideal for describing the effects of light on plants because plant pigments do not have the same relative sensitivities as the human eye to radiant energy of different wavelengths, and so it is better to define the light regimes experienced by plants in physical (energy) terms. Nevertheless illumination units have been widely used in early botanical literature (that on plant tissue culture being no exception). The following section therefore gives a brief description of the derivation of both illumination and energy units and how they may be approximately inter-converted for comparative purposes.

9.1. UNITS OF LIGHT MEASUREMENT

Energy units. Standard units (e.g. erg and joule) are used to quantify light energy. The amount of radiant energy emitted (or received) from a light source, per unit time, is termed the radiant flux. Radiant flux per unit area is known as the radiant flux density, and that intercepted per unit area, as irradiance (or the radiant flux density at a surface).

Quantum units. Radiant energy in wave form (including light), is emitted in discrete indivisible quantities called 'quanta' or 'photons'. The very small amount of energy in each quantum is inversely proportional to the wavelength of the radiation. Quantum units are particularly suited to the measurement of light where its energy may be utilised for photochemical reactions, especially photosynthesis. Quanta are measured in moles (the preferred SI unit), or Einsteins, one Einstein (E) being defined as the energy of 6.023×10^{23} (i.e. the number of molecules per gram molecule, Avogadro's number) photons. A substance must absorb one Einstein of radiant energy per mole to take part in a photochemical reaction. The energy of an Einstein varies inversely with the wavelength of light and is greatest in the blue and ultra-violet part of the spectrum. The quantum (or photon) flux density at a surface is equivalent to irradiance measured in standard energy units. The size of a photon flux is sometimes referred to as the rate of photon fluence.

Units relating to illumination. The luminous qualities of light are defined in a similar fashion to its radiant energy attributes. Luminous flux (the passage of light per unit time) is measured in lumens and luminous intensity (the candela). The candela now replaces the formerly accepted unit of visible light intensity, the candle. Intercepted visible light per unit area is termed illuminance (or illumination), corresponding to irradiance in energy terms. Illuminance (measured in lumens m^{-2} or lux) is thus luminous flux density at a surface. Under older terminology, illuminance was measured in foot candles. One foot candle was equivalent to one lumen per square foot.

Table 12.1 summarises these different categories of light units. It is only possible to convert between irradiance [or photon (quantum) flux density] and illuminance for any given wavelength of light. Exact factors for light sources emitting over many wavelengths are therefore impossible, and even average conversion factors will depend on the precise spectral emission. The interconversions between illuminance and energy units presented in Table 12.2 should therefore only be used to make approximate comparisons between dissimilar units reported in different scientific papers.

Table 12.1 Units and symbols used in the measurement of various light characteristics
[those units prefixed by a similar symbol are equivalent]

Characteristic	Unit	Symbol
Wavelength	Nanometer (1 nanometer = 10^{-9} metres)	nm
Energy	Joule Erg Einstein Calorie	J Erg E Cal
Energy flux	Watt	W
Luminous flux	□ Lumen □ Candela steradian	Lu cd.st
Radiant intensity	Watts per steradian	W/st
Luminous intensity	Candela (replace candle) Lumens per steradian	Cd (Candle = c) Lu/st
Illuminance (Illumination) (Luminous flux density at a surface)	Lumen per square foot [foot candle] ○ Lux ○ Lumen per square metre	Lu ft^{-2} Lx Lu m^{-2}
Incident radiant flux Irradiance (Radiant flux density at a surface) Quantum (Photon) flux density	 ‡ Watts per square metre ‡ Joules per square metre per second Ergs per meter square per second † Einsteins per square metre per second or † Moles (photons) per sq. metre per second	 W m-2 J m^{-2} s^{-1} ergs m^{-2} s^{-1} E m^{-2} s^{-1} mol m^{-2} s^{-1}

Table 12.2 Factors for converting between light units commonly found in the plant tissue culture literature

Units of Energy	1 erg = 1 x 10^{-7} joules 1 cal = 4.185 joules Energy of 1 E = 1.2 x $10^8/\lambda$ joules An Einstein (E) is 1 mole of photons, and the energy it provides therefore varies with the wavelength (λ) of the light in nm
Units of Illuminance	1 foot candle = 10.764 lux or lumen m^{-2} 1 candle (standard) = 0.02 Watts
Units of Luminous flux	1 candlepower = 4 π lumens
Units of Irradiance	1 erg cm^{-2} s^{-1} = 1 x 10^{-3} W m^{-2} 1 μmol m^{-2} s^{-1} = 1 μE m^{-2} s^{-1} = 0.215 W m^{-2} = 0.215 J m^{-2} s^{-1}

Note: Take reciprocals to convert in the opposite direction, e.g. 1 erg = 10^{-7} J and therefore 1J = $1/10^{-7}$ erg = 10^7 erg

9.3. THE LIGHT REQUIREMENTS OF PLANTS

The growth and development of plants is dependent on light for:

- Photosynthesis, the process whereby light energy is converted to chemical energy in the biosynthesis of chemicals from carbon dioxide and water.
- Photomorphogenesis, the light-induced development of structure or form.

This phenomenon does not necessarily involve the absorption of large amounts of light energy, and uses receptor systems which act as switching devices setting in motion the morphogenetic processes characteristic of light-grown plants. There are however some photo-morphogenetic systems which require prolonged exposure to high irradiance (HI) before they will occur.

- Phototropism, the growth response of plants which is induced by unilateral light. Photosynthesis, photomorphogenesis and photo-tropism are all facilitated by pigments in the tissues, which absorb radiation of particular wavelengths.

As explained in the section above on carbon dioxide, the photosynthesis carried out by most cultured plant material is relatively low, and cultures are mainly dependent on an external supply of sucrose. In these circumstances, light is most important for its effect on photomorphogenesis.

Phototropism is not of great relevance in plant tissue cultures.

9.3.1. Photosynthesis

Photosynthesis is energised by light of wavelengths between 400 and 700 nm, and light in this waveband is termed photosynthetically active radiation (PAR). The terms photosynthetic photon flux density (PPFD) or photosynthetic photon flux (PPF) are therefore often used to describe the photon flux density of 400-700 nm light.

9.3.2. Photomorphogenesis

Plants can follow two alternative developmental strategies depending on whether they are grown in the dark (skotomorphogenesis) or in the light (photomorphogenesis). In the dark, plants become etiolated, i.e. they invest their energy in rapid stem elongation; there is no leaf expansion, and a functional photosynthetic apparatus is not formed. Exposure to light induces a rapid change in gene expression leading to the normal pattern of development.

The signals, which lead to the photomorphogenic reactions of plants are subdivided according to the part of the spectrum which produces the response. The majority of responses are induced by either red or blue wavelengths. Red light-mediated systems have been extensively studied in plant material, and at least three photochromic pigments called phytochromes are now thought to act as receptors (Sharrock and Quail, 1989). Phytochromes not only absorb red light but also low wavelength ultra-violet light. Blue light receptors are less well understood. One pigment, primarily absorbing light of 450-480 nm wavelengths (the blue/UV-A part of the spectrum), has been termed cryptochrome; the other absorbs irradiation of wavelengths around 290-300 nm (the UV-B spectrum). Plant responses to light of the blue-UV regions of the spectrum can therefore be due to the effect of one receptor, or of two (e.g. a phytochrome and cryptochrome) or more, interacting together (Schäfer *et al.,* 1990). Sometimes the blue-UV sensory pigments need to be activated before a phytochrome response can be triggered (Mohr, 1987).

Photosynthetic pigments act as a light trap and pass on the energy they absorb to other molecules, which then facilitate the oxidation-reduction reactions leading to carbohydrate synthesis. A photochromic pigment like phytochrome, on the other hand, is one whose absorption properties change after exposure to light of a specific wavelength and threshold. In one form, Type II phytochrome has its absorption maximum in the red light region; upon exposure to red light the absorption maximum changes to the far-red region. Exposure of the pigment to far-red light while it is in this state, causes a high proportion of it to revert to the original form. This property of Type II phytochrome, which gives it a 'switching' capability, is often represented by the equation in Fig. 12.1. Action peak spectra for phytochrome *in vitro* have been found to be 10 nm lower than the above values (Smith and Kendrick, 1976).

The manner in which phytochrome pigments in their alternative forms regulate specific biochemical reactions has been investigated using a range of mutants and transgenics.

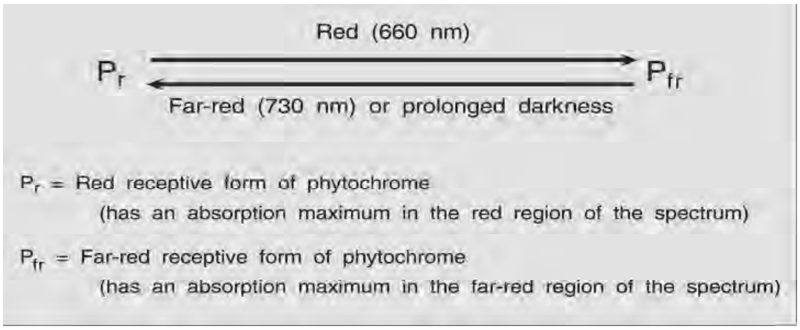

Fig. 12.1 The conversion of phytochrome into an active (P_{fr}) by red light, and reversal by far-red light.

10. THE INFLUENCE OF LIGHT ON TISSUE CULTURES

The three aspects of the photo-environment which most clearly influence *in vitro* growth and morphogenesis, are:

- wavelength;
- flux density; and
- the duration of light exposure or photoperiod.

Each of these attributes has an effect on both photomorphogenesis and photosynthesis. The influence can be direct, through an action on tissue already growing *in vitro*, or indirect through the influence of light on stock plants. In the latter case, the growth or morphogenesis displayed *in vitro* is modified by the light treatments applied to the stock plant before explants are removed. The effects of light on photosynthesis are not of great importance in tissue cultures, unless photoautotrophic growth is required.

The growth of organised plant tissues *in vitro* is not generally inhibited by light, which is often required for optimal results. On the other hand, initial cell divisions of explants and the growth of callus tissues are sometimes prevented by light.

10.1. FORMS OF LIGHTING FOR GROWTH ROOMS

Most micropropagation work is carried out under artificial lighting in temperature-controlled growth rooms.

Seibert *et al.* (1975) found that although growth and morphogenesis of tobacco callus was only influenced by low irradiance blue or near-U.V. monochromatic light, when tubes emitting white light (composed of many wavelengths) were compared, the best growth and shoot formation was obtained with tubes emitting a proportion of total light energy in the red region (600-700 nm). Similarly Fridborg and Eriksson (1975) discovered much lower hormone levels to be necessary for multiplication under light from white tubes (supplying 8 Wm^{-2}). These results suggest that red light can reinforce the stimulatory effect of blue and near-U.V. light when it is supplied simultaneously. Seedling explants of *Brassica* produced more adventitious shoots in Grolux light of 32 $\mu molm^{-2}s^{-1}$ than under 30 $\mu mol\ m^{-2}\ s^{-1}$ from white fluorescent tubes (Lazzeri and Dunwell, 1986b).

Fluorescent tubes. Fluorescent tubes are used almost universally to provide light for cultures. They have the advantage that shelves within a room can be conveniently illuminated without the creation of too great an amount of heat. Fluorescent lighting is sometimes supplemented with incandescent bulbs to ensure an adequate supply of wavelengths in the red end of the spectrum, but this is not always desirable. Micropropagation growth rooms are usually equipped with cool white (warm white), or Grolux tubes, or mixtures thereof (Seabrook, 1987). A mixture of cool-white and Agrolite (F40/AGO Westinghouse) gave plants from single node cuttings of potato which had the maximum leaf area, number of leaves, stem length and fresh and dry weight. Phillips TL-84 fluorescent lights produced plants with short internodes.

A mixture of Grolux and cool white tubes gave satisfactory rates of shoot proliferation of fruit tree cultures (Fiorino and Loreti, 1987): leaves of cultured shoots were said to be greener than under normal fluorescent lights (Rugini *et al.,* 1987), but Hasegawa *et al.* (1973) found no difference between the effectiveness of cool white or Grolux fluorescent lamps in promoting the formation of shoots from shoot cultures of *Asparagus*.

Other lighting sources. Bulblet formation on *Achimenes longifera* leaf and stem cuttings was promoted by high illuminance (15000-20000 lux) light from a mixture of Grolux and cool white tubes, but was strongly inhibited in light of the same luminous flux density from mercury vapour tungsten filament lamps (Deutch, 1974). A disproportionately high emission of far-red and infra-red light from the mercury vapour lamps seemed to be responsible for the inhibition. Incandescent lamps can also be a source of far-red irradiation. The profuse branching of *Xanthium strumarium* plants kept under fluorescent lights for 16 h each day, was prevented if the plants were given an additional 30 minutes illumination from tungsten filament bulbs. The inhibition was thought to have been caused by the far-red light content of the incandescent lighting (Tucker and Mansfield, 1972). Murashige and Nakano discovered incandescent lamps to be unsatisfactory for promoting organogenesis in tobacco (Hasegawa *et al.,* 1973).

To supply light at sufficiently high photon flux densities to support active photosynthesis of cultured shoots and plantlets, several research establishments (e.g. Kozai *et al.,* 1987; Lee *et al.,* 1988) have installed metal-halide or sodium vapour lamps, or have used them in conjunction with fluorescent lighting, in growth rooms. Norton *et al.* (1988; Norton and Norton, 1986) found that shoot proliferation in shoot cultures of woody plants was almost twice as great in the light from high pressure sodium discharge lamps as in that supplied by

fluorescent tubes. Plant growth under light from metal halide lamps was also greater than under fluorescent tubes, but less than under the sodium sources. Unfortunately, lamps supplying high energy light also emit considerable amounts of heat, and their use within growth rooms can necessitate considerable additional cooling costs unless the heat is channelled away by some means.

10.2. WAVELENGTH

10.2.1. Blue and near-U.V. light

Callus growth and morphogenesis. Callus of some species can be initiated and grown in the dark, while tissues of other plants grow better in continuous light or in a regular photoperiod (e.g. that of potato - Helgeson, 1980; tomato fruits - Mukund *et al.,* 1988).

By using sources emitting light over narrow bandwidths, it has been shown that near U.V. and blue light can have a controlling influence on the rate of growth and morphogenesis of some callus cultures. Callus initiated in the dark is often moved into the light when indirect morphogenesis is desired.

In a detailed study, Seibert *et al.* (1975) demonstrated that the combined effect of both wavelength and flux density can be important for callus growth and morphogenesis. Using tobacco callus, they showed that although the tissue would grow in the dark, its growth was stimulated, and shoot formation was promoted to the greatest extent by near-U.V. light (371 nm) at the very low irradiance of 0.24 Wm^{-2} (an illuminance of ca. 90 lux), but was inhibited when the incident flux density was more than 1.5 Wm^{-2} (ca. 540 lux); blue light of 420 or 467 nm wavelengths caused growth stimulation which was maximal at 3 and 6 Wm^{-2} (ca. 1080 and 2160 lux), respectively.

Other experiments have given conflicting results. Bergmann and Balz (1966) obtained stimulation of tobacco callus growth with blue light (435 nm) at an irradiance of 2.4 Wm^{-2} (ca. 860 lux) and Weis and Jaffe (1969) found that blue light of mixed spectral composition (15.5 Wm^{-2} or ca. 5600 lux), or white light, was essential for shoot bud development from tobacco callus. Red and green light were without effect. By contrast, in experiments of Ward and Vance (1968), even though callus growth of *Pelargonium* was particularly enhanced when cultures were kept in white and blue (450 nm) light, a stimulatory effect was also caused by light of other colours. *Crepis capillaris* callus increased in fresh weight more quickly in blue light than in darkness, but the greatest rate of growth occurred in red light (Husemann and Reinert, 1976). Red light has been noted to promote growth and organogenesis in cultures of some other species (see later).

Hedtrich (1977) obtained no root formation from leaf discs of *Prunus mahaleb* in darkness, a small number of roots in light from white fluorescent lamps (3000 lux), but more where some of the light was provided by lamps giving near-U.V. light.

Blue and near-U.V. responses are thought to be regulated by one or more pigments, which are particularly sensitive to light of these wavelengths. The situation is complicated, because phytochrome Pr and Pfr have minor absorption peaks at 365 nm (near-U.V.) and 495 nm (blue) respectively, and in some circumstances blue and near-U.V. light is capable of converting Pr into Pfr, but much less effectively than red light (Smith and Kendrick, 1976). Phytochrome also has strong absorbence at 280 nm (U.V.). This has been attributed to absorption by the inactive protein part of the molecule, energy then being transferred from the protein to the chromophore moiety.

Inhibition of callus growth. Inhibition of callus growth or cell division by blue light of higher incident flux densities than used by Seibert *et al.* (loc. cit.) has been reported on several other occasions, for example, in *Parthenocissus* (Klein, 1964), tobacco (Beauchesne and Poulain, 1966a,b), carrot (Polevaja, 1967), Jerusalem artichoke (Fraser *et al.,* 1967), *Pseudotsuga menziesii* (Kadkade and Jopson, 1978) and *Actinidia deliciosa* (Loreti *et al.,* 1991). The growth of *Haplopappus gracilis* callus was suppressed when cultures were moved from the dark to the light (Stickland and Sunderland, 1972a,b). When light of different wavelengths was compared at an irradiance of ca. 10 Wm^{-2} , blue had the greatest inhibitory effect; white and red light caused a similar but smaller reduction in the rate of cell division. Far-red light on the contrary, stimulated growth.

Fridborg and Eriksson (1975) found that callus or suspension cultures of *Nicotiana tabacum*, *Happlopappus gracilis*, haploid *N. sylvestris* and *Allium cepa*, were inhibited to varying degrees by continuous blue to near-U.V. light; but that the growth inhibition could be partially overcome by the cytokinin, 2-iP. For maximum growth, tobacco callus required 10 times more cytokinin in blue light than in white light.

Growth and embryogenesis of carrot suspensions was inhibited, but not prevented, by white (26 $\mu mol\ m^{-2}\ s^{-1}$) and blue (27.5 $\mu mol\ m^{-2}\ s^{-1}$) light, but the

highest number of embryoids was obtained when the suspensions were kept in the dark (or in red or green light, which produced the same response as dark treatment). Michler and Lineberger (1987) who obtained these results, suggested that red and blue light spectra could be used with advantage to manipulate carrot embryogenesis and embryo growth: as embryos grew, blue light also encouraged the formation of secondary embryos; red light hastened development towards the heart stage; while ABA synthesis at this time was most pronounced under blue light.

Possible causes of light-inhibited growth. The initial cell divisions in explants of artichoke tissue were found by Fraser *et al.* (1967) to be suppressed by light, and Yeoman and Davidson (1971) later showed that:

- cells were sensitised to light by 2,4-D (there was no effect in the absence of auxin);
- the cells have a light sensitive phase; exposure before or after this produced no response.

Alternative causes of growth inhibition induced by higher levels of blue or near-U.V. light might be:

- increased production of phenolic compounds which interfere with growth regulator activity (Andersen and Kasperbauer, 1973),
- destruction of cytochrome oxidase which is involved in respiratory processes (El-Mansy and Salisbury, 1971),
- enhanced biosynthesis of gibberellins (Beauchesne *et al.,* 1970). Evidence in favour of this hypothesis was obtained by Fridborg and Eriksson (1975), who found that the inhibitor of gibberellin synthesis, chlormequat (CCC), stimulated the growth of *Haplopappus gracilis* callus and cell suspensions grown in blue light. Cells and tissues of this plant were also inhibited by the addition to the medium of 0.1 mg/l gibberellic acid. However, in callus cultures of some other plants, gibberellic acid is found to stimulate growth.
- inhibition of natural cytokinin synthesis, or production of abscisic acid, which is antagonistic to cytokinin.

Growth inhibition by high incident flux densities of near-U.V. and blue light could also be caused by the accelerated metabolism of the auxin IAA (Fridborg and Eriksson, 1975) and may occur by the photo-degradation of this natural auxin in the presence of light-sensitised riboflavin (vitamin B2) (Galston and Hillman, 1961), or some other kind of flavonoid. Flavoprotein inhibitors can prevent callus growth in the light (Seibert *et al.,* 1975). Alternatively light may alter the oxidation of IAA by peroxidase enzymes, by regulating the levels of co-factors and inhibitors of the enzyme (Stickland and Sunderland, 1972b). Reductants such as ascorbic acid and polyphenols, inhibit the oxidation of IAA by riboflavin; possibly it is on account of the natural occurrence of these compounds, that some plant cells are able to grow and divide in the light. In most cultures, cells are stimulated to divide in the light by externally supplied auxins. Marcotrigiano and Stimart (1981) found that in the light, hypocotyls of *Paulownia* required 3 mg/l IAA in the medium to produce shoots at the maximum rate, whereas under continual darkness only 1 mg/l IAA was necessary. Cytokinin (kinetin, 3 mg/l) was required at the same concentration under both regimes. Fridborg and Eriksson (loc. cit.) made similar observations on shoot formation from callus.

The reaction of plant cells to light *in vitro* depends on their previous history. Light can inhibit callus colony formation from plated suspension cultured cells (Street, 1977b) and protoplasts obtained from cell suspensions invariably grow in the dark, but light is inhibitory (Reinert *et al.,* 1977). However, callus growth from mesophyll protoplasts takes place if they are first incubated in a low illumination (e.g. 300 lux) for a short while before being transferred to light of 3000 lux (Enzmann-Becker, 1973).

Blue light inhibition of axillary shoot proliferation. In a series of publications, Letouzé has described how the axillary buds of decapitated willow (*Salix babylonica*) cuttings remain dormant in blue light (Letouzé, 1970). The growth inhibition could be overcome by red light (or cytokinin treatment) and the red light promotion was reversed by subsequent exposure to blue light (Letouzé, 1974; 1975). In the presence of added growth substances, the light-mediated control of bud growth may not be apparent. A negative effect on shoot proliferation of fruit trees has been noted with light of 320-380 nm (Rugini *et al.,* 1987).

Behrouz and Lineberger (1981a,b) have investigated the interaction between white, blue, red or green light (15 μmol m^{-2} s^{-1}) and growth regulators, on the proliferation of shoots in shoot cultures of Juneberry. The regulants incorporated into the medium were 0.1 mg/l NAA + 2.5 mg/l BA. The greatest number of shoots was produced by using blue or white light together with the auxin and cytokinin.

Somewhat similar results were obtained by Norton *et al.* (1988; Norton and Norton, 1988b) in

shoot cultures of *Spiraea*. Without the presence of the cytokinin BA, these produced the same number of axillary shoots in red, blue or white (control) light. However there was significantly more shoot proliferation than in the control in red light when 0.25 mg/l BA was added to the medium, but less when the rate was increased to 0.5 mg/l. These results support the hypothesis that in many plants, omission of blue light (or irradiance with red - see below) can mimic or replace cytokinin dependency (Fig. 12.2). A few plants seem to behave differently. In the presence of 5 μM BA, axillary shoot proliferation of *Vitis* shoot cultures was greater in blue light than in red, especially if the medium contained only 5 μM Mn^{2+}, instead of 100 μM, as in MS medium (Chée, 1986).

U.V. light can induce specific enzyme activity. When chlorophyll-free cell cultures of parsley (*Petroselinum hortense*) are irradiated with light of a wavelength below 350 nm (particularly U.V. light, 290 nm), there are changes in the composition of messenger RNA. This leads to the induction of enzymes, which are specifically concerned with the formation of some particular flavonoid glycoside secondary products (Hahlbrock, 1977; Hahlbrock *et al.,* 1980).

10.2.2. The phytochrome system.

Seeds of some varieties of plants which are stimulated to germinate by red light, can alternatively be induced into growth by cytokinin treatment (Miller, 1956); but cytokinins are much more effective at promoting germination if seeds are kept in sufficient light to break their dormancy. Miller (1958) and Khan (1966) showed that with *Xanthium*, kinetin depended on a reversible phytochrome effect to overcome seed dormancy, its stimulatory effect being reversed by far red light. In other words, red light and cytokinin were complementary.

Kadkade and co-workers have reported on phytochrome systems in lettuce and pine. In hypocotyl callus cultures of the former, Kadkade and Seibert (1977) showed two phytochrome-sensitive systems to be in operation. Both callus growth and shoot organogenesis were greatly improved by daily 5 minute exposures to red light, and generally unaffected by similar far-red light treatments. The promotion caused by red light was reversed by afterwards placing the tissues in far-red light.

In most instances where red light specifically influences growth or morphogenesis in tissue cultures, it appears to have either the same effect as, or to reinforce, the addition of cytokinin to the medium. Red light, or polychromatic light containing a high proportion of red wavelengths, usually promotes axillary shoot growth (Fig 12.2). The response has been shown to be phytochrome dependent, because a short FR treatment at the end of a white light photoperiod can prevent branching (Tucker, 1976). Both red light and cytokinins promote bulblet formation in *Achimenes longifera* (Deutch, 1974).

Duckweed (*Lemna gibba*) cultured on a simple medium will not grow in the dark. The addition of a cytokinin will induce some growth as will periodic exposure to red light. Combining the cytokinin treatment (0.6 mg/l kinetin) with an eight-hourly exposure to 15 minutes of red light, produces the greatest growth response, the effect of which is reversed by far-red irradiation (Cleland, 1976). The promotive effect of red light on the growth of *Crepis capillaris* cells could be completely replaced by the addition of 1 mg/l kinetin to the medium, but kinetin had no effect on green cells cultured under blue light (Husemann and Reinert, 1976).

Shoot formation. Cultured root segments of *Convolvulus arvensis* showed enhanced shoot bud development under a light regime, which only provided 10 s of red light (655 nm) per day (at an irradiance of approximately 0.6 Wm^{-2}). This enhancing effect was reversed by far-red light (Bonnett, 1972). Under the same cultural conditions, the geotropic behaviour of roots of this weed was shown to be affected by the phytochrome system, as well as gravity. The roots grew horizontally in the dark, but showed a far-red reversible normal geotropic response on exposure to red light (Tepfer and Bonnett, 1972).

The involvement of phytochrome in shoot regeneration from callus was demonstrated by Bagga *et al.* (1985). When dark-grown leaf discs of *Brassica oleracea botrytis*, which had begun to form callus, were irradiated with only 5 minutes red light each day, they initiated shoots when transferred to continuous light. Explants, for which the red light treatment had been followed by 10 minutes far-red, did not. The cultures were grown throughout on Gamborg *et al.* (1968) B5 medium with 2 mg/l NAA and 0.5 mg/l *BA*.

An interaction between light and benzyladenine (BA) was demonstrated by Baraldi *et al.* (1988) for the promotion of shoot formation in *Prunus*. The number of shoots formed under blue, far-red and white light was highly dependent on the rate of photon fluence, but there was no difference in the rate of proliferation in different levels of red light,

suggesting that the BA induction of shoot formation is promoted by a low energy response of phytochrome.

The bulbing of tulip has been found to be enhanced under sources emitting light with a low red to far-red ratio (Alderson and Taeb, 1990).

Root formation. A phytochrome-mediated response was demonstrated in cultured pea roots by Furuya and Torrey (1964): low levels of incident red light would inhibit the initiation of lateral roots in cultured root segments, and the effect could be reversed by far-red wavelengths. In *Helianthus tuberosus* however, red light (optimum at around 660 nm) has been reported to induce adventitious root formation (Letouzé and Beauchesne, 1969). To explain these contrasting results, Thorpe (1980) suggested that the formation of lateral and adventitious roots might be influenced by light in different ways.

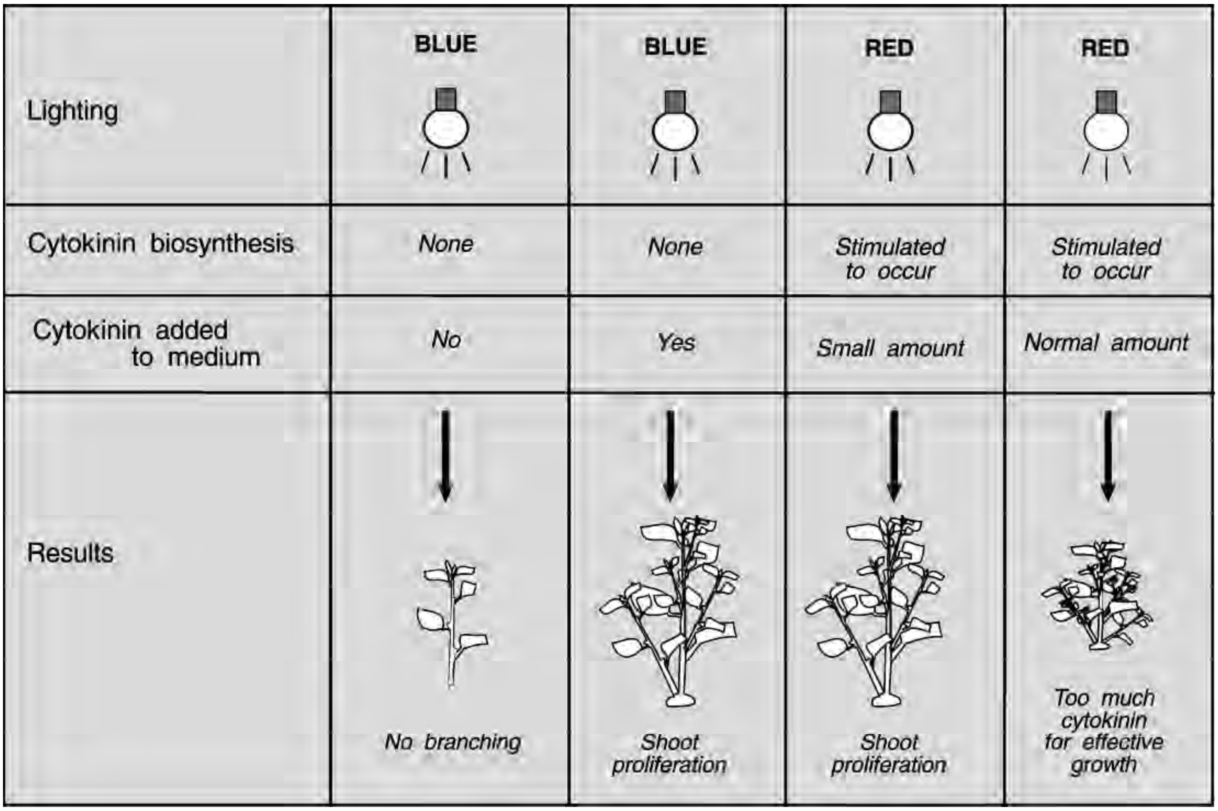

Fig. 12.2 An hypothesis to explain how the colour of light and cytokinin may interact to modify shoot proliferation.

Other work has tended to support a phytochrome-regulated stimulation of adventitious root formation. The *extra vitrum* rooting of azalea micro-cuttings was consistently better if shoot cultures had been kept for two weeks in far red light followed by 2 weeks of red illumination, than if the red light treatment preceded the far-red (Read and Economou, 1983). This result was confirmed by Economou (1986), who obtained enhanced rooting of micropropagated *Rhododendron* shoots by giving cultures 15 minutes red light at the end of a 14 h cool-white day. Chée and Pool (1989) obtained improved rooting of *Vitis* cuttings in red light or in fluorescent lighting supplying a relatively high proportion light in the red wavebands. Shoots of *Prunus* 'GF655/2' kept in far red light required auxin for *in vitro* rooting, but others kept in red light did not (Baraldi *et al.,* 1988).

Respiration. In protoplasts isolated from the leaf intercallary meristems of barley, respiration is partly controlled by phytochrome and a blue light receptor, blue light being able to reverse an inhibitory effect of abscisic acid (Owen *et al.,* 1987).

10.2.3. Green-Yellow light

Light in the yellow-red wavelengths has been found to promote shoot proliferation of fruit tree cultures (Rugini *et al.,* 1987; Loreti *et al.,* 1991). Tran Thanh Van *et al.* (1987) reported that 75% of the hypocotyl fragments of *Pseudotsuga menziesii* formed adventitious buds when cultured under monochromatic light of 550 nm (green/yellow), whereas only 35% of similar explants produced buds in white light (170 µmol m^{-2} s^{-1}), and light of 550 nm was found to suppress the growth of *Parthenocissus* crown gall callus (Klein, 1964). The combined results suggest that light of these wavelengths actively induces the destruction of auxin.

10.3. PHOTOSYNTHESIS

10.3.1. Light induced chlorophyll formation.

The development of chloroplasts. Chlorophyll, the green pigment in plants required for photosynthesis, is formed within the cell in organelles called chloroplasts. A chloroplast is one type of plant plastid, but plastids may become starch-containing (when they are called amyloplasts), or they may contain other coloured pigments (when they are called chromoplasts - found, for instance, in the cells of flowers and the skins of fruits), or oil, or stored protein. Given appropriate stimuli, amyloplasts can be converted to chloroplasts and vice versa. An undifferentiated plastid is sometimes called a 'pro-plastid'. Seedlings germinated in the dark contain plastids in their leaves, called etioplasts. These are already partly differentiated into chloroplasts and although they do not contain chlorophyll, they do possess some other accessory carotenoid pigments, one or more crystalline centres (called 'prolamellar bodies') and some other characteristics of a fully differentiated chloroplast. Buds induced to form from callus in the dark by high cytokinin concentrations, form etiolated shoot systems in which the plastids are similar to etioplasts (Stetler and Laetsch, 1968).

By contrast, in dark-grown cells of suspension or callus cultures, the plastids contain starch, do not have prolamellar bodies, and have only a few thylakoids without pigmentation (Bergmann and Berger, 1966; Stetler and Laetsch, 1968). (Thylakoids are series of membrane sacs on which chlorophyll is packaged within a chloroplast). In this respect tissue-cultured cells are akin to the undifferentiated cells of a potato tuber, where the plastids are amyloplasts and take several days to begin conversion into chloroplasts after the tubers are exposed to light.

Chlorophyll formation in tissue cultures. When callus or suspension cultures are transferred to light, they may develop chloroplasts and begin photosynthesis, but seldom become autotrophic. Greening generally proceeds very slowly compared to the rate in the etiolated shoots of intact plants, and cultures may take 8 weeks to reach maximum chlorophyll content. The ability to form chloroplasts seems to depend on the presence of non-dividing cells within cell aggregates, because when green callus tissue is used to initiate suspension cultures, chloroplasts de-differentiate and the level of pigment is reduced (Yeoman and Street, 1977). Sometimes the formation of green areas on otherwise colourless calluses is a first sign of the commencement of morphogenesis.

The chloroplasts formed in tissue cultured cells tend to be more variable in structure than those in leaf cells. They may not develop at a simultaneous rate and can possess aberrantly-shaped thylakoids (Davey *et al.,* 1971).

In a few plant species (e.g. some conifers), proplastids can become differentiated into chloroplasts in darkened buds (Sundqvist *et al.,* 1980). Most plants though, require some sort of light stimulus to initiate the differentiation of chloroplasts and the formation of chlorophyll. Several pigment systems may be involved. Light may be intercepted by:

- the chlorophyll precursor, protochlorophyllide that is present in dark-grown leaves;
- the phytochrome system;
- by the pigment system sensitive to blue and near-U.V. light.

Generally in whole plants, and also in most tissue cultures (Blackwell *et al.,* 1969), chlorophyll synthesis is particularly stimulated by red light. Protochlorophyllide has an absorption maximum at 634 nm and is converted by red light (or the red components of white light) to chlorophyll(ide) (Bjorn, 1976), the absorption maximum of phytochrome Pr being 650-660 nm. In tissue cultures, which may lack the biosynthetic precursors of chlorophyll, the phytochrome system is probably especially responsible for converting protochlorophyllide into chlorophyll (Satter and Galston, 1976) as it is in potato tubers (Morris *et al.,* 1979). However, some plants require light in the near-U.V. to blue, part of the spectrum (i.e. 370-450 nm) for the development of chloroplasts. The inductive effect of

blue light is then often enhanced by red light treatment before or afterwards (Sundqvist *et al.,* 1980).

Callus and suspension cultured cells of tobacco only develop chlorophyll in blue light (Bergmann and Berger, 1966; Kamiya *et al.,* 1981), which affects both the nuclear and plastid genomes (Richter and Wessel, 1985). In *Chenopodium rubrum*, blue light promotes changes in the transcription of genes that regulate the *de novo* synthesis of plastid and nuclear RNAs (Richter *et al.,* 1990). Chlorophyll retention of tobacco callus also requires blue light, for although photosynthesis occurs in green callus kept in red light, the quantity of chlorophyll in the tissues slowly declines (Bergmann and Balz, 1966)

Again emphasising the similarity between red-light and cytokinin-induced effects, cytokinins added to the medium have frequently been noted to promote chlorophyll development in callus or suspension cultures (Bandiera and Morpugo, 1970; Kaul and Sabharwal, 1971; El Hinnawiy, 1974) or even to be essential for chlorophyll formation in light (Stetler and Laetsch, 1968; Tandeau de Marsac and Peaud-Lenoel, 1972a,b). Formation of photosynthetic enzymes is also promoted by cytokinins, or independently by continuous far-red light (Feierabend, 1969).

10.4. DAYLENGTH (PHOTOPERIOD)

Daylength influences plants in two ways:

- By regulating the amount of radiant energy intercepted. Growth of photosynthetically dependent plants is proportional to the length of time that they are exposed to natural sunlight or artificial light;
- Through a controlling mechanism whereby plants are able to recognise changes in the environment. For this purpose plants are able to sense changes in the duration of light provided each day (photoperiod).

Genuine photoperiodic effects on plants are energised by light of relatively low irradiance (ca. 0.3 Wm^{-2}, Cathey and Campbell, 1980) and are generally associated with the phytochrome system.

Where cultured plant organs or tissues are not autotrophic, and are grown under lighting of relatively low irradiance, the effects of varying daylength on *in vitro* cultures are most likely to be due to photoperiod. However, total intercepted light energy may govern the biosynthesis of key metabolic products in green cultures (e.g. in shoot cultures) even though the level of chlorophyll is low and photosynthesis insufficient to sustain normal growth. As the total light energy supplied by photoperiods of different intervals is variable when provided by light of the same flux density, it is not always possible to be sure which of the two mechanisms mentioned above is responsible for many effects described in the literature.

The most well known effect of photoperiod is the regulation of flowering in many plant species. Short day plants such as chrysanthemum, require to be exposed to short days (i.e. 8-10 h of light and 14-16 h of darkness per day) for flowering to be induced, while in long day plants (e.g. wheat and barley) flowering is only initiated when they are kept in 14-16 h of light per day. There are many variations on this pattern, including species where flowering takes place independently of day length (e.g. tomato).

As with other plant responses that are naturally regulated by light, some of the effects which daylength might have on morphogenesis, may be replaced by additions of synthetic growth regulators to the culture medium. Sometimes however, light of the correct daylength is indispensable: the induction of flower formation in cultured shoot apices or stem explants is the best example, and here, the same photoperiodic treatments as would cause flowering to occur in the intact plant, have generally been found to be essential *in vitro*.

Murashige (1978) pointed out that plants needing specific photoperiods for vegetative growth and development are very likely to manifest this need during *in vitro* culture. However, the photoperiod requisite for flowering may not be the same as that necessary for optimum regeneration of shoots and roots, or for shoot proliferation. For example, although entire plants of *Kalanchoe pinnata* flower under short days, they naturally produce foliar plantlets under long days. Shoot cultures of cucumber (a short day plant) develop maximum foliation under long days (Rute *et al.,* 1978).

Both the formation and growth of vegetative organs may be influenced by the daylength under which tissue cultures are maintained. Some examples, which seem to be related to photoperiod are provided here, and other instances illustrating the effects of light and dark on morphogenesis have been placed in the section on irradiance that follows.

10.4.1. Daylength effects in shoot cultures

Benefit from long days. Haramaki (1971) observed that gloxinia plantlets regenerated from shoot cultures were etiolated and had small leaves when given 8 h illumination of 1075 lux daily. Longer 16 h daylengths gave thicker, stouter and darker leaves,

similar to those produced on plants grown under 3200 lux. Shoot cultures of *Pelargonium* have been established and encouraged to form multiple shoots in continuous fluorescent light of 500 lux (Debergh and Maene, 1977) and seedling shoot tips of *Pharbitis nil* (a short day plant) also grew and produced plantlets equally well in long days of 16 or 24 hours (Bapat and Rao, 1977). In 8 or 12 h photoperiods, shoot and root growth was inhibited. Roots grew well in the dark however, although of course in these conditions the shoots were etiolated. By contrast, apple shoot cultures required 16 h light of 60 μmol m^{-2} s^{-1} fluence for the maximum rate of shoot proliferation. The internode length of 'Delicious', 'Triple Delicious' and 'Vermont Spur Delicious' was less in 24 h than in 16 h days, although that of 'Empire' was greater (Yae *et al.,* 1987).

Short day plants. Shoot cultures of 'Rougen' grapevine had a requirement for short days. They grew, and later shoots formed roots, only in 10 h days, soon becoming necrotic when the daylength was 15, 16 or 24 h (Chée and Pool, 1982a,b). Where, as in *Camellia japonica*, shoot multiplication in shoot cultures is as rapid in a 12 h day as in one of 18 h, propagation under the shorter photoperiod can lead to a saving in lighting costs (Samartin, 1989). However, although *Dianthus caryophyllus* can be propagated in 12 h photoperiods, Stimart (1986) reported that in commercial practice, shoots have been found to flower *in vitro* under these conditions: most shoots are vegetative in a 16 h day.

Interaction with temperature. An interaction between temperature and daylength was observed by Rahman and Blake (1988) in *Artocarpus heterophyllus* shoot cultures grown under light of 85 μmol m^2 s^{-1}. An optimum number of axillary shoots was produced at 25°C in either a 12 or a 16 h day, but the maximum dry weight of harvested shoots occurred in a 12 h day at a temperature of 30°C. Photosynthesis appeared to contribute to growth, because shoot production was much less in the dark or in an 8 h day.

10.4.2. Adventitious shoot formation

Although shoot morphogenesis is generally stimulated by light, the most favourable daylength can vary from one genus to another. The following examples illustrate the kind of variation, which is experienced. In few, if any, of the cited experiments was it discovered whether the observed effects of daylength were due to photoperiod, or the total irradiance received by the cultures.

The formation of adventitious shoots from twin bulb scales of onion was more rapid in long days than in short days (Hussey and Falavigna, 1980), and this seems to be the case in lily too, where continuous light enhances shoot and root formation, and darkness promotes bulb growth (Stimart and Ascher, 1978). Under a PPF of 160 μmol $m^{-2}s^{-1}$ at 25°C, meristematic explants of *Capsicum annuum* formed adventitious shoots much more rapidly under a daylength of 12 h, than in 16 h days, or in continuous light (Phillips and Hubstenberger, 1985). Similarly, in the experiments of Pillai and Hildebrandt (1969), the callus of *Pelargonium* did not turn green and form adventitious buds in continuous high illumination (21500 lux from Grolux tubes and incandescent lamps). Shoot regeneration was optimal when these callus cultures were maintained in a photoperiod of 15-16 h. By contrast, shoot regeneration from *Brassica* callus was greater in continuous light than in a daylength of 16 hours (6000 lux, 20°C) (Jain *et al.,* 1988) and shoots formed directly from leaf segments of *Peperomia scandens* in continuous light of 2700 lux (Kukulczanka *et al.,* 1977).

Heide (1964) found that adventitious shoots were produced more freely from begonia leaf cuttings when the cuttings themselves and/or the mother plants from which they had been taken, were kept in short days. Callus derived from the anther wall of *Hevea* only gave rise to somatic embryos when grown under 8 h days or when transferred from a 16 h to an 8 h photoperiod (Wan Abdul *et al.,* 1981), and although the formation of *Pyrus serotina* callus was not affected by daylength, shoot morphogenesis was promoted by short days or culture in the dark (Hiratsuka and Katagiri, 1988).

10.4.3. Adventitious root formation

The rooting of cuttings taken directly from stock plants is influenced by the length of the days in which the plants have been grown. Effects due to total incident radiation (availability of stored carbohydrates) and daylength (photomophogenesis) can be seen (see Chapter 11).

10.5. ILLUMINANCE AND IRRADIANCE ('LIGHT INTENSITY')

Intensity is a property of a light source and, strictly according to physical definitions, describes its radiant or luminous flux per solid angle. The word 'intensity' is however colloquially used to describe either luminous or radiant flux density and appears widely in early plant tissue culture literature, especially when the physiological effects of light

were thought to be due to the total amount of energy that had been incident on an organ or tissue, rather than to daylength or phytochrome-controlled processes.

'Intensity' influences the total light energy received by plant cultures. The induction of chlorophyll formation in plant organs or callus or cell cultures, is accompanied by increased activity (or *de novo* synthesis) of key enzymes in photosynthesis (Koth, 1974) although, as explained previously, the level of carbon fixation which normally occurs in callus cultures is well below that of leaf mesophyll cells. However, the improved growth of some types of green callus in light can be due to the enhanced availability of photosynthetically derived carbohydrate (Bergmann and Balz, 1966).

Daylight may provide irradiance of ca. 200-700 Wm^{-2} (equivalent to an illuminance of ca. 50000-150000 lux, see Table 12.3), but plant cultures are typically grown in flux densities, which are about 10 times less than daylight and have a different spectral composition. There are several reasons why low irradiance light is used:

- provision of high levels of artificial lighting is expensive and generates unwanted heat;
- cultures in sealed vessels become overheated in high irradiances due to the glasshouse effect (Pierik, 1987);
- the technology of plant tissue culture has evolved using non-autotrophic tissues supplied with a carbohydrate.

As lighting technology advances there is more argument for using higher irradiances and allowing plants to develop more autotrophic growth (e.g. Zobayed *et al.,* 2000). There is evidence that good quality plantlets can be produced and that some of the problems associated with the micropropagation technique can be avoided (Kozai *et al.,* 1997).

Table 12.3 A comparison between units of Incident Radient Flux. Approximate conversion factors are given below for three fluorescent light sources are commonly used in plant tissue culture growth rooms [after McCree, 1972a,b]. To convert from lux (units of illuminance) into Irradiance or Quantum Flux Density units, divide lux by the following figures, depending on the type of fluorescent lighting tube

Units	Warm white	Cool white	Grolux
$W\ m^{-2} = J\ m^{-2}\ s^{-1}$	390	370	280
$mW\ cm^{-2}$	3900	3700	2800
$\mu W\ cm^{-2}$	3.9	3.7	2.8
$mW\ cm^{-2}$	39	37	28
$erg\ mm^{-2}\ s^{-1}$	39	37	25
$erg\ cm^{-2}\ s^{-1}$	0.39	0.37	0.25
$\mu mol\ m^{-2}\ s^{-1}$ ($\mu E\ m^{-2}\ s^{-1}$)	83	80	59
$\mu mol\ cm^{-2}\ s^{-1}$ ($\mu E\ cm^{-2}\ s^{-1}$)	$8.3x10^5$	$8.0x10^5$	$5.9x10^5$

10.5.1. Callus growth

Callus of many plants can be initiated and maintained in the dark and it has been said that there is no point in culturing tissues in the light if they do not contain chlorophyll. Indeed the callus of some plants may not grow at all in the light, or its growth may be severely inhibited (see above). In cultures intended for indirect shoot regeneration, callus is often initiated on explants in the dark at Stage I, and is then moved to the light at Stage II when organogenesis is to be instigated.

However, some callus tissues grow better in the light than in the dark. In geranium an optimum level of incident light for the growth of callus depended on the variety of plant from which stem explants were derived (Hammerschlag, 1978). In two varieties, growth was best when the tissues were illuminated with 1000 or 5000 lux, in another it was most rapid only in 5000 lux. In all three varieties growth was reduced when the callus was kept in 10000 lux. Inhibition of callus growth by high illuminance could be associated with increased production of growth inhibitors. The production of phenolic compounds is stimulated by light in callus cultures of some species (particularly those lacking chlorophyll), and greater amounts are accumulated as illuminance is increased (Davies, 1972).

10.5.2. Shoot growth and proliferation

From experiments with shoot tip propagation of several different kinds of plants, Murashige (1974) suggested that an optimum level of illumination for Stages I and II might be 1000 lux (i.e. approximately 14-15 $\mu mol\ m^{-2}\ s^{-1}$). From the literature, it seems

that, at these stages, cultures of many herbaceous plants are maintained in photon flux densities of 7-120 μmol m^{-2} s^{-1} (1.5-9.5 Wm^{-2}; equivalent to an illuminance of about 0.5-10 klux). However, culture initiation under low irradiance may help to prevent blackening in the cultures of sensitive species.

Plants of many genera (e.g. aster, chrysanthemum and the Caryophyllaceae) require relatively high irradiances for optimum shoot multiplication, and culture in low lighting causes shoots to become succulent and vitreous (hyperhydric), and hence of poor quality. *Hosta* shoot cultures require high light to commence proliferation, but thereafter are able to tolerate light of both high and low irradiance (Stimart, 1986). *Brunnera macrophylla* callus must be kept in complete darkness for adventitious shoot formation (Stimart, 1986). In some plants, shoot proliferation in shoot cultures, or direct shoot formation from explants, is enhanced by using high levels of irradiation at Stage II. Haramaki (1971) for instance, thought that *Sinningia* shoot cultures were best maintained at 3000-10000 lux (ca. 40-150 μmol m^{-2} s^{-1}); in lower illumination the shoot tips formed callus. Dalton and Dale (1981) found that more tillers were produced from *Lolium multiflorum* in 12000 lux (ca. 35 Wm^{-2}) than in 9600 lux, provided that BA was present in the medium.

High lighting levels are also required for some woody species. Axillary shoot proliferation in shoot cultures of an *Ulmus* hybrid required an irradiance of ca. 9.7 Wm^{-2} (45 μmol m^{-2} s^{-1}), but higher (possibly 14, and certainly 36.5 Wm^{-2}) were inhibitory to shoot development (Fink *et al.,* 1986). However, in *Castanea* shoot cultures, multiplication was most rapid at 27°C with 33 Wm^{-2} irradiance, and the best shoot elongation occurred at 34°C in 22 Wm^{-2} (Chauvin and Salesses, 1988). McGranahan *et al.* (1987) reported using 9.7-12.0 Wm^{-2} (that is. 45-60 μmol $m^{-2}s^{-1}$) for *Juglans*, while Evers (1984) obtained an optimum rate of growth of *Pseudotsuga menziesii* under 36.4 Wm^{-2} or 24.5 Wm^{-2}, depending on the time of year at which isolations were made.

Cultures needing low irradiance. There are plants sensitive to high light *in vitro* and these can be multiplied satisfactorily in light of relatively low fluence: *Dicentra spectabilis* is an example. *Gloxinia* plantlets obtained through shoot culture were progressively larger and their leaf size greater, when illuminated with about 3200 lux, but in 10700 lux there was very little growth and leaves were small and discoloured. Callus was present in 320 lux, but absent at the higher flux densities (Haramaki, 1971).

Miller and Murashige (1976) showed that the rate of shoot proliferation of *Cordyline terminalis* was greatest in an illumination of only 300 lux, whereas three other tropical species of foliage ornamentals multiplied most rapidly in 3000 or 10000 lux. Cultures of *Phalaenopsis* orchids produced axillary shoots more freely from flower stalk cuttings in 0.25 Wm^{-2} than in 2.34 Wm^{-2} irradiance (Tanaka *et al.,* 1988), but the rate of shoot multiplication in *Lythrum virgatum* cultures was no different in 0.3-3.2 Wm^{-2} (Heuser, 1983).

Although Einset and Alexander (1985), Hildebrandt and Harney (1983) and Welander (1987) had mainly grown *Syringa* plants at 8-10 Wm^{-2}, Pierik *et al.* (1986; 1988a) utilised 4-5 Wm^{-2} for single node cultures of *S. vulgaris*, because stems were more elongated at this irradiance. Shoots grown in 1-3 Wm^{-2} had thin stems and very small leaves. In practical micropropagation work, it would be important to ascertain that any increased shoot production at higher lighting levels was cost effective.

Although the multiplication of axillary shoots of asparagus was satisfactory in 1000 lux, higher illuminance of 3000-10000 lux was essential at Stage III to cause shoots to initiate needle-like cladophylls. An illumination of 30000 lux was excessive (Hasegawa *et al.,* 1973). Zygotic embryos of *Populus deltoides* grown in reduced light produced more vigorous shoots than those grown at higher light intensities (Kouider *et al.,* 1984).

Storage. Where light is necessary for the storage of cultures, a PPF of 10-15 μmol $m^{-2}s^{-1}$ is usually employed.

10.5.3. Morphogenesis

Flower buds. The formation of floral buds (rather than vegetative shoot buds) on cultured explants has usually been found to require the presence of light. In pieces of cold-treated chicory root, the inductive action of light took place during the first few days of culture and presumably during bud initiation. Very thin stem explants of tobacco consisting of just a few cell layers similarly required light to form flower buds, and unless light was supplied during days 7-10 of culture they formed vegetative shoot buds instead (Tran Thanh Van, 1980; Trinh and Tran Thanh Van, 1981). The formation of tobacco flower buds in this system appears to be promoted by long days and high flux densities (Tran Thanh Van, 1977). Red light was also more promotive than far-red light or darkness.

Adventitious shoots. Adventitious shoot formation can take place in the dark, but it is usually greater in the light; for example, more shoots were produced directly from strawberry leaf discs or from a short intervening callus phase, in light of 1000 lux illuminance, than in the dark (Nehra *et al.,* 1988).

Direct shoot formation in endive leaf fragments was reduced by a short period of darkness during culture (Legrand, 1972). Continuous darkness was especially inhibitory if interrupted by 3 days of continuous 600 lux fluorescent light at the beginning or after the first 3 days (Legrand, 1974). A similar situation has been discovered in *Heloniopsis orientalis*, where morphogenesis was inhibited in darkness unless one of several sugars was added to the medium during the first few days of treatment (Kato, 1978a). From these results it seemed that shoot formation was dependent on photosynthesis, an hypothesis which appeared to be confirmed when it was found that in the absence of sugar, bud initiation was inhibited in CO_2-free air and by chemical inhibitors of photosynthesis. White, red, blue and far-red light were effective promoters, but not green light (Kato, 1978b).

Shoot regeneration from seedling leaf discs of *Brassica oleracea* did not appear to be influenced by photosynthesis, because more shoots were produced at 30 μmol $m^{-2}s^{-1}$ than at 100 or 200 μmol $m^{-2}s^{-1}$. The reverse was true for root segments (Lazzeri and Dunwell, 1986b).

Adventitious roots. The formation of adventitious roots on cuttings seems to be affected by light energy. In many plants, the induction of roots is promoted by darkness or light of low fluence, apparently because natural auxin levels increase in these conditions, whereas they are decreased by light of high fluence. For root formation and root growth though, cuttings or explants require high energy inputs, and supplying mother plants with supplementary illumination, culturing cuttings with sucrose, or placing cuttings with root primordia in high light prior to root induction, can all promote root growth.

Root formation from green photosynthetic *Cymbidium* protocorms appeared to depend on how they were illuminated. In the dark, or in light of ca. 1250 lux, protocorms gave rise only to shoots, whereas both roots and shoots were formed in ca. 2200-2500 lux (Ueda and Torikata, 1972). It was thought that root formation was associated with nitrogen metabolism which was enhanced as illuminance and photosynthesis increased.

Cotyledons of *Cucumis melo* cultured in the light formed either shoots or morphogenic callus, whereas those cultured in the dark, formed either roots or friable non-morphogenic callus (Mackay and Ng, 1988).

Morphogenesis in callus cultures. Light is not always essential for adventitious shoot formation from callus, although sprouting is frequently enhanced under illuminated conditions (e.g. in *Anthurium* - Pierik *et al.,* 1974b). A strong correlation between carbohydrate availability and organogenesis in callus cultures has been reported (Mangat *et al.,* 1990), and when optimum levels of sugars are provided, light may have only a regulatory effect (i.e. it is not required for photosynthesis). Shoot regeneration from *Arabidopsis* callus on an induction medium was enhanced if the tissue had been previously cultured in continuous low illuminance (less than 1000 lux), rather than in darkness, but higher illuminance (more than 4000 lux) greatly reduced the percentage of regenerating calluses (Negrutiu and Jacobs, 1978). Callus of tobacco grown on Lin and Staba (1961) medium, supplemented with 15% coconut milk and 2 mg/l kinetin, was induced to form somatic embryos by high illuminance (10000-15000 lux) (Haccius and Lakshmanan, 1965).

10.5.4. Flux densities for other stages of micropropagation

As with other aspects of the cultural environment, light 'intensity' at each propagation stage may need to be varied for particular plants or for methods of micropropagation other than shoot culture. Unfortunately, the most suitable level can vary even from one cultivar to another. In *Begonia hiemalis* adventitious shoot formation of one variety was improved when cultured in 6000 lux rather than 3000 lux, but two other varieties did not respond in this way (Welander, 1978).

For the micropropagation of deciduous azaleas, Economou and Read (1986) found that 30-75 μmol $m^{-2}s^{-1}$ was better than 10 μmol $m^{-2}s^{-1}$. During the shoot multiplication stage 10-75 μmol $m^{-2}s^{-1}$ was required (although 75 μmol $m^{-2}s^{-1}$ produced shoots of less good quality); the highest percentage of rooted shoots was obtained when Stage III cultures were kept under an irradiance of 10 μmol $m^{-2}s^{-1}$.

An illuminance of 3000-10000 lux (ca. 40-150 μmol $m^{-2}s^{-1}$) was suggested by Murashige (1974) for Stage III to achieve maximum rates of transfer of shoots or plantlets to an exterior environment, and

many laboratories find that 40-75 μmol $m^{-2}s^{-1}$ is adequate. In lower flux densities shoots may be etiolated with pale green leaves. Daylengths of 16 h are usually employed.

Sweet gum plants grown under high irradiances (50-315 μmol $m^{-2}s^{-1}$ from metal halide lamps) either *in vivo* or *in vitro*, were found to have larger cells and a more compact mesophyll than those grown in low light. But all *in vitro* plants had smaller, thinner leaves and smaller mesophyll cells than *in vivo* plants, and except at 315 μmol $m^{-2}s^{-1}$, significantly greater stomatal densities (Lee *et al.,* 1988).

High light levels can be beneficial for special purposes, such as germinating seedlings from which explants are to be excised (Lazzeri and Dunwell, 1986a,b), or germinating somatic embryos (McGranahan *et al.,* 1987), when irradiances of ca. 135-200 μmol m^{-2} s^{-1} may be necessary.

10.6. DARKNESS

Although light is invariably essential for the growth of normal green shoots and plantlets, unorganised cell and tissue cultures can frequently be grown in its absence, and darkness may be beneficial to growth and morphogenesis. Darkness is also used to etiolate shoots. Treating light-grown seedlings to 2-3 days darkness can sometimes help to elongate hypocotyls or epicotyls before explants are excised (Jagannathan and Marcotrigiano, 1986).

Excised green leaves of *Heloniopsis* grown in the light tend to produce a preponderance of shoot initials at the apex (distally). In darkness however, most green and etiolated leaves tended to develop buds at the base (Kato, 1978a). Scale sections of *Lilium longiflorum* regenerated a small number of large bulblets in the dark, each having only a few leaves. Bulblets produced in illuminated cultures were small and bore many leaves, and leaf dry weight was double that of the bulbs (Leshem *et al.,* 1982).

A short period of darkness sometimes promotes shoot morphogenesis. Adventitious buds did not form on swollen needle primordia of *Picea pungens* unless cultures were kept in the dark for 8 days. Subsequent shoot development required 16 h under 1000 lux illumination (Misson *et al.,* 1982).

10.6.1. Establishing cultures

As explained in Chapter 2, keeping cultures in the dark at Stage I can help in establishing explants. This is primarily because visible blackening of the tissues is reduced, although sometimes the formation of less obvious growth inhibitors may also be prevented.

10.6.2.. Callus growth

Callus growth does not take place in some plants unless light is excluded from cultures, for example *Hosta* scape explants only produce callus in the dark (Stimart, 1986). *Magnolia* shoot tip callus ceased to grow if moved to even low light and would not resume growth until transferred twice to fresh medium during a 6 week period (Biederman, 1987). In other species, light may be necessary for callus initiation, but once callus has been obtained, it can be subcultured and maintained in the dark. Where light causes shoot formation to occur [e.g. in *Anthurium scherzerianum*, Geier (1986)], shoot development may be at the expense of callus growth and so callus induction and multiplication may need to be carried out in the dark.

10.6.3. Morphogenesis

Light is often necessary for the formation of adventitious shoots from callus or explants, but this is not always the case. In *Freesia hybrida*, the requirement for light or dark varied with the plant variety. Callus from young ovaries of some cultivars could produce shoots in the dark, while that from other cultivars required to be incubated in the light (Bach, 1987). Bhatt *et al.* (1979) failed to regenerate shoots from *Solanum khasianum* in the light, but did obtain shoots and roots in the dark.

Sometimes light controls the type of morphogenesis which takes place. Callus from the gametophyte generation of the moss *Physcomitrium coorgense*, only regenerated sporophytes if cultured in the dark, but gave rise to both sporophytes and gametophytes in diffuse daylight (Lal, 1963). Leaf explants of *Solanum melongena* produced callus and roots in the dark, but rooted shoots when cultures were moved to the light (Macchia *et al.,* 1983).

However, in some species caulogenesis or embryogenesis can occur in continuous darkness. For example, fragments of the corms of *Crocus sativus* cultured in darkness on an agar medium, formed miniature corms, which also proliferated (Homs *et al.,* 1987). Sometimes darkness is essential to morphogenesis; for example, scape explants of *Hosta* only form callus and adventitious shoots in the dark (Stimart, 1986). Embryogenic callus was seen on young leaflets of *Euphorbia longan* after 2 months culture in the dark. Embryos grew to maturity after transfer to another medium in the dark, but required 16 h light per day for germination. There was precocious germination if embryos were exposed to light prematurely (Litz, 1988).

A brief period of darkness. Occasionally morphogenesis can be enhanced if explants are kept in the dark for a certain period. The formation of adventitious buds on young flower buds of *Freesia* was greatly improved if the explants were grown in the dark before being transferred to the light (Pierik and Steegmans, 1975). Similarly, keeping segments of apple leaves in the dark for the first three weeks of culture, strongly enhanced the subsequent regeneration of adventitious shoots and embryo-like structures (Welander, 1988). Meyer (1982) and Dai *et al.* (1987) found that more shoots were formed from *Rhododendron* floral explants if they were cultured in the dark for at least two weeks, before being moved to the light.

Maintaining anther cultures of some species in the dark for an initial period can increase the proportion of anthers producing somatic embryos or the frequency with which anther-derived callus is obtained (Sunderland and Roberts, 1977; Sangwan-Norreel, 1977; Nair *et al.,* 1983).

10.6.4. Rooting

Treatment of mother plants or cuttings with a period of darkness, or light of very low fluence, frequently favours the initiation of adventitious roots on cuttings.

REFERENCES

AITKEN J., HORGAN K.J. & THORPE T.A. 1981 Influence of explant selection on the shoot-forming capacity of juvenile tissues of *Pinus radiata*. Can. J. Forest Res. 11, 112- 117.

AITKEN-CHRISTIE J. & JONES C. 1987 Towards automation: radiata pine shoot hedges *in vitro*. Plant Cell Tissue Organ Cult. 8, 185-196.

AKITA M. & TAKAYAMA M. 1988 Mass production of potato tubers using jar fermentor techniques. Acta Hortic. 230, 55-61.

ALDERSON P.G. & TAEB A.G. 1990 Influence of cultural environment on shoot growth and bulbing of tulip *in vitro*. Acta Hortic. 266, 91-94.

ALPI A. & GARIBALDI M.I. 1969 Propagation by vegetative tip culture in a species of *Cymbidium*. Riv. Ortoflorofruttic. Ital. 53, 159-167.

ANDERSEN R. & KASPERBAUER M.J. 1973 Chemical composition of tobacco leaves altered by near-ultraviolet and intensity of visible light. Plant Physiol. 51, 723-726.

APPELGREN M. & HEIDE O. 1972 Regeneration in *Streptocarpus* leaf discs and its regulation by temperature and growth substances. Physiol. Plant. 27, 417-423.

BACH A. 1987 The capability of *in vitro* regeneration of various cultivars of *Freesia hybrida*. Acta Hortic. 212, 715-718.

BAGGA S., RAJASEKHAR V. K., BANDYOPADHYAY S. & CHATTOPADHYAY S.B. 1985 Enhancement in the formation of shoot initials by phytochrome in stem callus cultures of *Brassica oleracea* var. *botrytis*. Plant Science 38, 61-64.

BANDIERA M. & MORPUGO G. 1970 Kinetin/auxin ratio and development of chloroplasts in tissue cultures of *Daucus carota*. Experientia 26, 558-559.

*BA*AT V.A. & RAO P.S. 1977 Shoot apical meristem culture of *Pharbitis nil*. Plant Sci. Lett. 10, 327-334.

BARALDI R., ROSSI F. & LERCARI B. 1988 *In vitro* shoot development of *Prunus* GF655-2: interaction between light and benzyladenine. Physiol. Plant. 74, 440-443.

BARLASS M. & SKENE K.G.M. 1982 A scanning electron microscope study of adventitious bud formation in the grapevine. pp. 159-160 in Fujiwara A. (ed.) 1982 (q.v.).

BARRETT-LENNARD E.G. & DRACUP M. 1988. A porous agar medium for improving the growth of plants under sterile conditions. Plant Soil 108, 294-298.

BARTON L.V. 1956 Growth response of physiologic dwarfs of *Malus arnoldiana* Sarg. to gibberellic acid. Contrib. Boyce Thomps. 18, 311-317.

BATESON J.M., GROUT B.W.W. & LANE S. 1987 The influence of container dimensions on the multiplication rate of regenerating plant cell cultures. pp. 275-277 in Ducaté *et al.* (eds.) 1987 (q.v.).

BARZ W., REINHARD E. & ZENK M.H. (eds.) 1977 *Plant Tissue Culture and its Biotechnological Application.* Springer-Verlag, Berlin, Heidelberg, New York.

BEAUCHESNE G. & POULAIN C. 1966a Influence des éclairements approximativement monochromatiques sur la développement des tissus, de moëlle de tabac cultivés *in vitro* en présence d'auxine et de kinétine. Photochem. Photobiol. 5, 157-167.

BEAUCHESNE G. & POULAIN C. 1966b Quelques observations sur le développement des cultures *in vitro* de moëlle de tabac en présence d'auxine et de kinétine, en éclairement relativement monochromatique. Photochem. Photobiol. 5, 385-389.

BEAUCHESNE G., POULAIN C. & LETOUZÉ R. 1970 Les cultures *in-vitro* de tissu de moëlle de tabac W.38 en éclairements monochromatiques. Photo-inactivation des auxines indoliques. Physiol. Plant. 23, 1101- 1109.

BEHROUZ M. & LINEBERGER R.D. 1981a Influence of light quality on *in vitro* shoot multiplication of *Amelanchier laevis*. HortScience 16, 406.

BEHROUZ M. & LINEBERGER R.D. 1981b Interactive influences of auxins and cytokinins on proliferation of cultured *Amelanchier* shoot tips. HortScience 16, 453.

BERGMANN L. & BALZ A. 1966 Der Einfluss von Farblicht auf Wachstum und Zusammensetzung pflanzlicher Gewebe-kulturen. Planta 70, 285-303.

BERGMANN L. & BERGER C.H. 1966 Farblicht und Plastidendifferenzierung in Zellkulturen von *Nicotiana tabacum* var. `Samsun'. Planta 69, 58-69.

BHATT P.N., BHATT D.P. & SUSSEX I.M. 1979 Organ regeneration from leaf discs of *Solanum nigrum*, *S. dulcamara*. and *S. khasianum*. Z Pflanzenphysiol 95, 355-362.

BIEDERMANN I.E.G. 1987 Factors affecting establishment and development of *Magnolia* hybrids *in vitro*. Acta Hortic. 212, 625-629.

BJORN L.O. 1976 The state of protochlorophyll and chlorophyll in corn roots. Physiol. Plant. 37, 183-184.

BLACK C.C. Jr. & HUMPHREYS T.E. 1962 Effects of 2,4-dichlorophenoxyacetic acid on enzymes of glycolysis and pentose phosphate cycle. Plant Physiol. 37, 66-73.

BLACKWELL S.J., LAETSCH W.M. & HYDE B.B. 1969 Development of chloroplast fine structure in aspen tissue culture. Am. J. Bot. 56, 457-463.

BLAKE J. & EEUWENS C.J. 1982 Culture of coconut palm tissues with a view to vegetative propagation. pp. 145-148 *in* Rao A.N. (ed.) 1982 (q.v.).

BONGA J.M. 1977a. Application of tissue culture in forestry, pp. 93-108. *in* Reinert J. and Bajaj Y. P. S. (eds.) 1977 *Plant Cell, Tissue and Organ Culture*. Springer-Verlag, Berlin.

Bonga, J. M. 1977b. Organogenesis in *in vitro* cultures of embryonic shoots of *Abies balsamea* (Balsam fir). In Vitro 13: 41–48.

BONGA J.M. AND DURZAN D.J. (eds.) 1987 *Cell and Tissue Culture in Forestry. Vol. 2. Specific Principles and Methods: Growth and Development*. Martinus Nijhoff Publishers, Dordrecht, Boston, Lancaster. ISBN 90-247-3431-2.

BONNER J. & AXTMAN G. 1937 The growth of plant embryos *in vitro*. Preliminary experiments on the role of accessory substances. Proc. Natl Acad. Sci. USA 23, 453-457.

BONNETT H.T. 1972 Phytochrome regulation of endogenous bud development in root cultures of *Convolvulus arvensis*. Planta 106, 325-330.

BROWN D.C.W. & THORPE T.A. 1980 Changes in water potential and its components during shoot formation in tobacco callus. Physiol. Plant. 49, 83-87.

BROWN D.C.W. 1988 Germplasm determination of *in vitro* somatic embryogenesis in alfalfa. HortScience 23, 526-531.

BUWALDA F. & GREENWAY H. 1989 Nitrogen uptake and growth of wheat during O_2 deficiency in root media containing NO_3^- only, or NO_3^- plus NH_4^+. New Phytol. 111, 161-166.

CARMAN J.G. 1988 Improved somatic embryogenesis in wheat by partial stimulation of the *in-ovulo* oxygen growth regulator and desiccation environments. Planta 175, 417-424.

CASSELLS A.C. & MINAS G. 1983 Plant and *in vitro* factors influencing the micropropagation of *Pelargonium* cultivars by bud-tip culture. Sci. Hortic. 21, 53-65.

CATHEY H.M. & CAMPBELL L.E. 1980 Light and lighting systems for horticultural plants. Hortic. Rev. 2, 491-537.

CHALUPA V. 1987 Temperature. pp. 142-151 in Bonga J.M. and Durzan D.J. (eds.) 1987 *Cell and Tissue Culture in Forestry. Vol. 1. General Principles and Biotechnology*. Martinus Nijhoff Publishers, Dordrecht, Boston, Lancaster. ISBN 90-247-3430-4.

CHAUVIN J.E. & SALESSES G. 1988 Advances in chestnut micropropagation (*Castanea* sp.). Acta Hortic. 227, 340-345.

CHEE R. & POOL R.M. 1982a The effects of growth substances and photoperiod on the development of shoot apices of *Vitis* cultured *in vitro*. Sci. Hortic. 16, 17-27.

CHEE R. & POOL R.M. 1982b *In vitro* micropropagation of *Vitis*, results with species and cultivars. HortScience 17, 530.

CHEE R. & POOL R.M. 1989. Morphogenic response to propagule trimming, spectral irradiance and photoperiod of grapevine shoots cultured *in vitro*. J. Am. Soc. Hort. Sci. 114, 350-354.

CHEE R. 1986 *In vitro* culture of *Vitis*: The effects of light spectrum, manganese sulfate and potassium iodide on morphogenesis. Plant Cell Tissue Organ Cult. 7, 121-134.

CHENG T.-Y. 1976 Organogenesis in culture from cotyledon explants of Douglas fir. Plant Physiol. 57, Suppl., 66.

CHENG T.-Y. 1978 Clonal propagation of woody species through tissue culture techniques. Comb. Proc. Int. Plant Prop. Soc. 28, 139-155.

CHENG Y.W. & CHUA S.E. 1982 The use of air flow system in plant tissue and organ cultures. pp. 210-212 in Rao A.N. (ed.) 1982 (q.v.).

CLELAND C.F. 1976 Synergistic interaction of red light and cytokinin in promotion of growth in *Lemna gibba* G3. Plant Physiol. 57, Suppl., 75.

CORNEJO-MARTIN M.J., MINGO-CASTEL A.M. & PRIMO-MILLO E. 1979 Organ redifferentiation in rice callus: effects of C_2H_4, CO_2 and cytokinins. Z. Pflanzenphysiol. 94, 117-123.

CRAWFORD R.M.M. & BRAENDLE R. 1996. Oxygen depri-vation stress in a changing environment. J. Exp. Bot. 47, 145-159.

DAI C., LAMBETH V.N., TAVEN R. & MERTZ D. 1987 Micropropagation of *Rhododendron prinophyllum* by ovary culture. HortScience 22, 491-493.

DALTON C.C. & STREET H.E. 1976 The role of the gas phase in the greening and growth of illuminated cell suspension cultures of spinach (*Spinacea oleracea* L.). In Vitro 12, 485-494.

DALTON S.J. & DALE P.J. 1981 Induced tillering of *Lolium multiflorum in vitro*. Plant Cell Tissue Organ Cult. 1, 57-64.

DAVEY M.R., FOWLER M.W. & STREET H.E. 1971 Cell clones contrasted in growth, morphology and pigmentation isolated from a callus culture of *Atropa belladonna* var. *lutea*. Phytochemistry 10, 2559-2575.

DAVIES M.E. 1972 Polyphenol synthesis in cell suspension cultures of Paul's Scarlet Rose. Planta 104, 50-65.

DAVIES W.J. & SANTAMARIA J.M. 2000 Physiological markers for microplant shoot and root quality. Acta Hortic. 530, 363-369.

DAVIES W.J., WILKINSON S. & LOVEYS B. 2002 Stomatal control by chemical signalling and the exploitation of this mechanism to increase water use efficiency in agriculture. New Phytol. 153, 449-460.

DAVIS M.J., BAKER R. & HANAN J.J. 1977 Clonal multi-plication of carnation by micropropagation. J. Am. Soc. Hortic. Sci. 102, 48-53.

DEBERGH P. & MAENE L. 1977 Rapid clonal propagation of pathogen-free pelargonium plants starting from shoot tips and apical meristems. Acta Hortic. 78, 449-454.

DEBERGH P.C. 1986a Micropropagation in horticulture. Proc. of Inst. Hort. Symposium, Univ. Nottingham, School of Agric. UK.

DEBERGH P.C. 1986b Plant tissue and cell culture. pp. 383-393 *in* Green C.E. *et al.* (eds.) Alan R. Liss Inc., N.Y.

DEBERGH P.C. 1988 Micropropagation of woody species -state of the art on *in vitro* aspects. Acta Hortic. 227, 287-295.

DEUTCH B. 1974 Bulblet formation in *Achimenes longiflora*. Physiol. Plant. 30, 113-118.

DIAMANTOGLOU S. & MITRAKOS K. 1979 Sur la culture *in vitro* de l'embryon d'olivier (*Olea europea* L. var. Oleaster). Compt. Rend. Acad. Sci. Paris 288D, 1537-1540.

DUCATÉ, G., JACOB M. AND SIMEON A. 1987 (eds.). *Plant Micropropagation in Horticultural Industries. Preparation, Hardening and Acclimatization Processes*. Belgian Plant Tissue Culture Group, Symposium Florizel 87; Arlon, Belgium.

DRIVER J.A. & SUTTLE G.R.L. 1987 Nursery handling of propagules. pp. 320-335 in Bonga and Durzan (eds.) 1987 (q.v.).

DUNWELL J.M. 1981 *In vitro* regeneration from excised leaves of three *Brassica* species. J. Exp. Bot. 32, 789-799.

EARLE E.D. & LANGHANS R.W. 1974a Propagation of carnation *in vitro*. HortScience 9, 270-271.

EARLE E.D. & LANGHANS R.W. 1974b Propagation of *Chrysanthemum in vitro*. II. Production growth and flowering of plantlets from tissue cultures. J. Am. Soc. Hortic. Sci. 99, 352-358.

EARLE E.D. & LANGHANS R.W. 1975 Carnation propagation from shoot tips cultured in liquid medium. HortScience 10, 608-610.

EARNSHAW B.A. & JOHNSON M.A. 1985 The effect of gluta-thione on development in wild carrot suspension cultures. Biochem. Biophys. Res. Commu. 133, 988-993.

EARNSHAW B.A. & JOHNSON M.A. 1986 The role of antioxidants in the growth and development of wild carrot suspension cultures. p. 340 in Somers *et al.* (eds.) 1986 (q.v.).

ECONOMOU A. & READ P.E. 1986 Influence of light duration and irradiance on micropropagation of a hardy deciduous azalea. J. Am. Soc. Hortic. Sci. 111, 146-149.

ECONOMOU A.S. 1986 Effect of red and far-red light on azalea microcutting production *in vitro* and rooting *in vivo*. p.431 in Somers *et al.* (eds.) 1986 (q.v.).

EINSET J.W. & ALEXANDER J.H. (III) 1985 Multiplication of Syringa species and cultivars in tissue culture. Comb. Proc. Int. Plant Prop. Soc. 1984 34, 628-636.

EL HINNAWIY E. 1974 Effect of some growth regulating substances and carbohydrates on chlorophyll production in *Melilotus alba* (Desr.) callus tissue cultures. Z. Pflanzenphysiol. 74, 95-105.

EL-MANSY H.I. & SALISBURY F.B. 1971 Biochemical responses of *Xanthium* leaves to ultraviolet light. Rad. Bot. 11, 325-328.

ENZMANN-BECKER G. 1973 Plating efficiency of protoplasts of tobacco in different light conditions. Z. Naturforsch. 28, 470-471.

EREZ A., COUVILLON G.A. & KAYS S.J. 1980 The effect of oxygen concentration on the release of peach leaf buds from rest. HortScience 15, 39-41.

EVERETT N. 1982 The determination phase of differentiation. pp. 93-94 *in* Fujiwara (ed.) 1982 (q.v.).

EVERS P.W. 1984 Growth and morphogenesis of shoot initials of Douglas fir, *Pseudotsuga mensiesii* (Mirb.) Franco, *in vitro*. Diss. Agric. Univ. Wageningen.

FAVRE J.-M. 1977 Premiers résultats concernant l'obtention *in vitro* néoformations culinaires chez la vigne. Ann. Amel. Plant. 27, 151-169.

FEIERABEND J. 1969 Der Einfluss von Cytokinen auf die Bildung von Photosyntheseenzymen in Roggenkeimlingen. Planta 84, 11-29.

FINK C.V.M., STICKLEN M.B., LINEBERGER R.D. & DOMIR S.C. 1986 *In vitro* organogenesis from shoot tip, internode and leaf explants of *Ulmus* `Pioneer'. Plant Cell Tissue Organ Cult. 7, 237-245.

FIORINO P. & LORETI F. 1987 Propagation of fruit trees by tissue culture in Italy. HortScience 22, 353-358.

FITTER, A.H. & HAY, R.K.M. 2001 Enviromental physiology of plants. Elsevier, Amsterdam.

FLEMION F. 1959 Effect of temperature, light and gibberellic acid on stem elongation and leaf development in physiologically dwarfed seedlings of peach and *Rhodotypos*. Contrib. Boyce Thompson Inst. 20, 57-70.

FONNESBECH M. 1974a The influence of NAA, BA and temperature on shoot and root development from *Begonia cheimantha* petiole segments grown *in vitro*. Physiol. Plant. 32, 49-54.

FONNESBECH M. 1974b The influence of naphthyl acetic, benzyl ademine and temperature on plantlet development from petiole segments of *Begonia cheimantha* grown *in vitro*. Proc. Int. Hortic. Cong. 19, 66.

FOYER C., ROWELL J. & WALKER D. 1983 Measurement of the ascorbate content of spinach leaf protoplasts and chloroplasts during illumination. Planta 157, 239-244.

FRASER R.S.S., LOENING U.E. & YEOMAN M.M. 1967 Effect of light on cell division in plant tissue cultures. Nature 215, 873.

FRIDBORG G. & ERIKSSON T. 1975 Partial reversal by cyto-kinin and (2-chloroethyl)-trimethylammonium chloride of near-ultraviolet inhibited growth and morphogenesis in callus cultures. Physiol. Plant. 34, 162-166.

FUCHIGAMI L.H. & NEE C.-C. 1987 Degree growth stage model and rest-breaking mechanisms in temperate woody perennials. HortScience 22, 836-845.

FUJIWARA A. (ed.) 1982 *Plant Tissue Culture* 1982. Proc. 5th. Int. Cong. Plant Tiss. Cell Cult., Japan. Assoc. Plant Tissue Culture, Japan.

FUKAMI T. & HILDEBRANDT A.C. 1967 Growth and chlorophyll formation in edible green plant callus tissues *in vitro* on media with limited sugar supplements. Bot. Mag. Tokyo 80, 199-212.

FURUYA M. & TORREY J.G. 1964 The reversible inhibition by red and far-red light of auxin-induced lateral root initiation in isolated pea roots. Plant Physiol. 39, 987-991.

GALSTON A.W. & HILLMAN W.S. 1961 The degradation of auxin. Encyclopedia of Plant Physiology 14, 647-670.

GAMBORG O.L., MILLER R.A. & OJIMA K. 1968 Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151-158.

GATHERCOLE R.W.E., MANSFIELD K.J. & STREET H.E. 1976 Carbon dioxide as an esssential requirement for cultured sycamore cells. Physiol. Plant. 37, 213-217.

GEIER T. 1986 Factors affecting plant regeneration from leaf segments of *Anthurium scherzerianum* Schott (Araceae) cultured *in vitro*. Plant Cell Tissue Organ Cult. 6, 115-125.

GERTSSON U.E. 1988 Development of micropropagated plants from different clones of *Senecio hybridus* in relation to BAP concentration and temperature *in vitro*. J. Hortic. Sci. 63, 489-496.

GORTER C.J. 1965 Vegatative propagation of *Asparagus officinalis* by cuttings. J. Hortic. Sci. 40, 177-179.

GRAVES R.H., REID R.H. & MATHES M.C. 1978 Tissue culture of the monocots *Lilium*, *Hemerocallis* and *Hippeastrum*. p. 248 in Hughes *et al.* (eds.) 1978 (q.v.).

GREWAL S., KOUL S., AHUJA A. & ATAL C.K. 1979 Horm-onal control of growth, organogenesis and alkaloid production in *in vitro* cultures of *Hyoscyamus muticus* Linn. Ind. J. Exp. Biol. 17, 558-561.

GRUNEWALDT J. 1988 *In vitro* selection and propagation of temperature tolerant *Saintpaulia*. Acta Hortic. 226, 271-275.

HACCIUS B. & LAKSHMANAN K.K. 1965 Adventive embryo-genesis in callus culture of *Nicotiana* under high light intensity. Planta 65, 102-104.

HAHLBROCK K. 1977 Regulatory aspects of phenylpropanoid biosynthesis in cell cultures. pp. 95-111 *in* Barz, Reinhard and Zenk (eds.) 1977 (q.v.).

HAHLBROCK K., SCHRODER I. & VIEREGGE J. 1980 Enzyme regulation in parsley and soybean cell cultures. pp. 39-60 *in* Fiechter A. (ed.) 1980 *Advances in Biochemical Engin-eering. Vol 18.* Springer-Verlag, Berlin, Heidelberg, New York.

HAMMERSCHLAG F.A. 1982 Factors affecting establishment and growth of peach shoots *in vitro*. HortScience 17, 85-86.

HAMMERSCHLAG F.A. 1978 Influence of light intensity and date of explantation on growth of geranium callus. HortScience 13, 153-154.

HAMMERSCHLAG F.A. 1981 Factors affecting the culturing of peach shoots *in vitro*. HortScience 16, 282 (Abst.).

HARADA H. & IMAMURA J. 1983 Factors that stimulate pollen embryogenesis. pp. 145-158 *in Cell and Tissue Culture Tech-niques for Cereal Crop Improvement.* Science Press, Beijing, China and Gordon Breach Science Publishers Inc., New York.

HARAMAKI C. 1971 Tissue culture of *Gloxinia*. Comb. Proc. Int. Plant Prop. Soc. 21, 442-448.

HASEGAWA P.M., MURASHIGE T. & TAKATORI F.H. 1973 Propagation of aparagus through shoot apex culture. II. Light and

temperature requirements, transplantability of plants, and cyto-histological characteristics. J. Am. Soc. Hortic. Sci. 98, 143-148.

HATANO K., KAMURA K., SOYAMA Y. & NISHIOKA I. 1988 Clonal multiplication of *Aconitum carmichaeli* by tip culture and alkaloid contents of clonally propagated plant. Planta Med. 54, 152-155.

HEDRICH C.M. 1977 Differentiation of cultivated leaf discs of *Prunus mahaleb*. Acta Hortic. 78, 177-183.

HEIDE O.M. 1964 Effects of light and temperature on regenerative ability of *Begonia* leaf cuttings. Physiol. Plant. 17, 789-804.

HEIDE O.M. 1965 Interaction of temperature, auxins and kinins in the regeneration ability of *Begonia* leaf cuttings. Physiol. Plant. 18, 891-920.

HELGESON J.P. 1980 Plant tissue and cell suspension culture. pp. 19-25 in Ingram D.S. and Helgeson J.P. (eds.) 1980 *Tissue Culture Methods for Plant Pathologists*. Blackwell Scientific Publications.

HENRICH J.E., STIMART D.P. & ASCHER P.D. 1981 Terrestrial orchid seed germination *in vitro* on a defined medium. J. Am. Soc. Hortic. Sci. 106, 193-196.

HEUSER C.W. 1983 *In vitro* propagation of *Lythrum virgatum*. HortScience 18, p. 303.

HIGUCHI H. & AMAKI W. 1989 Effects of 6-benzylaminopurine on the organogenesis of *Asplenium nidus* L. through *in vitro* propagation. Sci. Hortic. 37, 351-359.

HILDEBRANDT A.C. & HARNEY P.M. 1983 *In vitro* propagation of *Syringa vulgaris* 'Vesper'. HortScience 18, 432-434.

HILDEBRANDT A.C., WILMAR J.C., JOHNS H. & RIKER A.J. 1963 Growth of edible chlorophyllous plant tissues *in vitro*. Am. J. Bot. 50, 248-254.

HIRATSUKA S. & KATAGIRI M. 1988 Shoot and callus formation in explants from immature seeds of Japanese pear. Sci. Hortic. 34, 193-199.

HOMS J., LEGROS M. & JAZIRI M. 1987 *In vitro* multiplication of *Crocus sativus* L. Acta Hortic. 212, 675-676.

HORN W., SCHLEGEL G. & JOHN K. 1988 Micropropagation of roses (*Rosa* hybr.). Acta Hortic. 226, 623-626.

HOWARD B.H. & OEHL V.H. 1981 Improved establishment of *in vitro*-propagated plum micropropagules following treatment with GA_3 or prior chilling. J. Hortic. Sci. 56, 1-7.

HUGHES K.W., HENKE R. & CONSTANTIN M. (eds.) 1978 *Propagation of higher plants through tissue culture.* Technical Inf. Center, U.S. Dept. Energy. Spingfield, Va. CONF-7804111

HUSEMANN W. & REINERT J. 1976 Regulating growth and morphogenesis of cell cultures of *Crepis capillaris* by light and phytohormones. Protoplasma 90, 353-367.

HUSSEY G. & FALAVIGNA A. 1980 Origin and production of *in vitro* adventitious shoots in the onion *Allium cepa* L. J. Exp. Bot. 31, 1675-1686.

HUSSEY G. & STACEY N.J. 1981 *In vitro* propagation of potato (*Solanum tuberosum* L.). Ann. Rep. John Innes Inst. 1980 57-59.

HUSSEY G. 1976 *In vitro* release of axillary shoots from apical dominance in monocotyledonous plantlets. Ann. Bot. 40, 1323-1325.

HUSSEY G. 1977 *In vitro* propagation of some members of the Liliaceae, Iridaceae, and Amaryllidaceae. Acta Hortic. 78, 303-309.

HVOSLEF-EIDE A. K. & PREIL W. 2005. *Liquid Culture Systems for in vitro Plant Propagation*. Springer, Dordrecht.

JACKSON G.V.H., BALL E.A. & ARDITTI J. 1977 Tissue culture of taro, *Colocasia esculenta* (L.) Schott. J. Hortic. Sci. 52, 373-382.

JACKSON M.B., ABBOTT A.J., BELCHER A.R. & HALL K.C. 1987 Gas exchange in plant tissue cultures. pp. 57-71 *in* Jackson M.B., Mantell S.H. and Blake J. *Advances in the Chemical Manipulation of Tissue Cultures*. Monograph 16, British Plant Growth Regulator Group, Bristol.

JACKSON M.B., ABBOTT A.J., BELCHER A.R., HALL K.C., BUTLER R. & CAMERON J. 1991 Ventilation in plant tissue cultures and the effect of poor aeration on ethylene and carbon dioxide accumulation, oxygen depletion and explant development. Ann. Bot. 67, 229-237.

JACKSON M.B., SAKER L.R., CRISP C.M., ELSE M.A. & JANOWIAK F. 2003. Ionic and pH signalling from roots to shoots of flooded tomato plants in relation to stomatal closure. Plant Soil 253, 103-113.

JAGANNATHAN L. & MARCOTRIGIANO M. 1986 Phenotypic and ploidy status of *Paulownia tomentosa* trees regenerated from cultured hypocotyls. Plant Cell Tissue Organ Cult. 7, 227-236.

JAIN R.K., CHOWDHURY J.B., SHARMA D.R. & FRIEDT W. 1988 Genotypic and media effects on plant regeneration from cotyledon explant cultures of some *Brassica* species. Plant Cell Tissue Organ Cult. 14, 197-206.

JAMES D.J., PASSEY A.J. & MALHOTRA S.B. 1984 Organogenesis in callus derived from stem and leaf tissues of apple and cherry rootstocks. Plant Cell Tissue Organ Cult. 3, 333-341.

JOHANSSON L., ANDERSSON B. & ERIKSSON T. 1982 Improvement of anther culture: activated charcoal bound in agar medium in combination with liquid medium and elevated CO_2 concentration. Physiol. Plant. 54, 24-30.

JOHNSON B.B. & MITCHELL E.D. 1978 The effect of temperature on *in vitro* propagation of *Broccoli* from leaf rib and leaf explants. In Vitro 14, 334.

JONES A.M. & PETOLINO J.F. 1988 Effects of support medium on embryo and plant production from cultured anthers of soft-red winter wheat (*Triticum aestivum* L.). Plant Cell Tissue Organ Cult. 12, 253-261.

JONES L.H. 1974 Long-term survival of embryoids of carrot (*Daucus carota* L.). Plant Sci. Lett. 2, 221-224.

JOY R.W., PATEL K.R. & THORPE T.A. 1988 Ascorbic acid enhancement of organogenesis in tobacco callus. Plant Cell Tissue Organ Cult. 13, 219-228.

JULLIEN M. 1974 La culture *in vitro* de cellules du tissu foliaire d'*Asparagus officinalis* L.: obtention de souches à embryogenèse permanente et régénération de plantes entières. Compt. Rend. Acad. Sci. Paris 279D, 747-750.

KADKADE P.G. & JOPSON H.1978 Influence of light quality on organogenesis from the embryo-derived callus of Douglas fir (*Pseudotsuga menziesii*). Plant Sci. Lett. 13, 67–73.

KADKADE P. & SEIBERT M. 1977 Phytochrome-regulated organogenesis in lettuce tissue culture. Nature 270, 49-50.

KADKADE P.G. & JOPSON H.A. 1977 *In vitro* studies of adventitious bud formation in Douglas fir and pine embryo cultures. Plant Physiol. 59, Suppl., 62.

KAMIYA A., IKEGAMI I. & HASE E. 1981 Effects of light on chlorophyll formation in cultured tobacco cells. I. Chlorophyll accumulation and phototransformation of protochlorophyll(ide) in callus cells under blue and red light. Plant Cell Physiol. 22, 1385-1396.

KAO K.N. 1981 Plant formation from barley anther cultures with Ficoll media. Z. Pflanzenphysiol. 103, 437-443.

KAPIK R.H., EARNSHAW B.A., CARLSON J.A. & JOHNSON M.A. 1986 The promotion of somatic embryogenesis by exogenous hydrogen peroxide. p. 372 *in* Somers *et al.* (eds.) 1986 (q.v.).

KATAEVA N.V. & BUTENKO R.G. 1987 Clonal micropropagation of apple trees. Acta Hortic. 212, 585-588.

KATO Y. & OZAWA N. 1979 Adventitious bud formation on leaf and stem segments of *Heloniopsis orientalis* grown at various temperatures. Plant Cell Physiol. 20, 491-497.

KATO Y. 1978a Induction of adventitious buds on undetached leaves, excised leaves, and leaf fragments of *Heloniopsis orientalis*. Physiol. Plant. 42, 39-44.

KATO Y. 1978b The involvement of photosynthesis in inducing bud formation on excised leaf segments of *Heloniopsis orientalis* (Liliaceae). Plant Cell Physiol. 19, 791-799.

KAUL K. & SABHARWAL P.S. 1971 Effects of sucrose and kinetin on growth and chlorophyll synthesis in tobacco tissue cultures. Plant Physiol. 47, 691-695.

KESSELL R.H.L. & CARR A.H. 1972 The effect of dissolved oxygen concentrations on growth and differentiation of carrot (*Daucus carota*) tissue. J. Exp. Bot. 23, 996-1007.

KHAN A.A. 1966 Breaking of dormancy in *Xanthium* seeds by kinetin mediated by light and DNA-dependent RNA synthesis. Physiol. Plant. 19, 869-874.

KLEIN R.M. 1964 Repression of tissue culture growth by visible and near visible radiation. Plant Physiol. 39, 536-539.

KONINGS H. & JACKSON M.B. 1979 A relationship between rates of ethylene production by roots and the promoting or inhibiting effects of exogenous ethylene and water on root elongation. Z. Pflanzenphysiol. 92, 385-397.

KOTH P. 1974 Änderungen in Enzymmuster ergrüender Kalluskulturen von *Nicotiana tabacum* var.'Samsun'. Planta 120, 207-211.

KOUIDER M., SKIRVIN R.M., SALADIN K.P., DAWSON J.O. & JOKELA J.J. 1984 A method to culture immature embryos of *Populus deltoides in vitro*. Can. J. Forest Res. 14, 956-958.

KOZAI T., KUBOTA C. & WATANABE I. 1988 Effects of basal medium composition on the growth of carnation plantlets in auto- and mixo-trophic tissue culture. Acta Hortic. 230, 159-166.

KOZAI T., OKI H. & FUJIWARA K. 1987 Effects of CO_2 enrichment and sucrose concentration under high photosynthetic photon fluxes on growth of tissue-cultured *Cymbidium* plantlets during the preparation stage. pp. 135-141 *in* Ducaté *et al.* (eds.) 1987 (q.v.).

KOZAI T., KUBOTA C. & JEONG B.R. 1997. Environmental control for large-scale production of plants through *in vitro* techniques. Plant Cell Tissue Organ Cult. 51, 49-56.

KRATZ K.A. & LANGHANS R.W. 1978 Tissue culture propagation and dormancy studies on *Clematis* cultivars. HortScience 13, 356.

KRIKORIAN A.D. & KANN R.P. 1979 Clonal micropropagation of daylilies. pp. 835-836 *in* Sharp W.R., Larsen P.O., Paddock E.F. & Raghavan V. (eds.) *Plant Cell and Tissue Culture*. Ohio State Univ. Press, Columbus.

KRUL W.R. & WORLEY J.F. 1977 Formation of adventitious embryos in callus cultures of `Seyval', a French hybrid grape. J. Am. Soc. Hortic. Sci. 102, 360-363.

KUKULCZANKA K., KLIMASZEWSKA K. & PLUTA H. 1977 Regeneration of entire plants of *Peperomia scandens* from different parts of leaves *in vitro*. Acta Hortic. 78, 365-371.

KUMAR P.P., REID D.M. & THORPE T.A. 1986 Ethylene and carbon dioxide accumulation and morphogenesis in excised cotyledons of *Pinus radiata*. p. 11 in Abstracts VI Intl. Cong. Plant Tissue & Cell Culture, Minneapolis, Minn.

LAL M. 1963 Experimental induction of apogamy and the control of differentiation in gametophytic callus of the moss *Physcomitrium coorgense* Broth. pp. 363-381 in Maheshwari P. and Ranga Swamy N.S. (eds.) *Plant Tissue and Organ Culture*. Int. Soc. Plant Morphologists, Delhi, India.

LANE W.D. 1982 Plant manipulation *in vitro* with hormones. Comb. Proc. Int. Plant Prop. Soc. 1981 31, 101-108.

LANGTON F.A. & COCKSHULL K.E. 1997. Is stem extension determined by DIF or by absolute day and night temperatures? Sci. Hortic. 69, 229-237.

LAZAR M.D., COLLINS G.B. & VIAN W.E. 1983 Genetic and environmental effects on the growth and differentiation of wheat somatic cell cultures. J. Hered. 74, 353-357.

LAZZERI P.A. & DUNWELL J.M. 1986a *In vitro* regeneration from seedling organs of *Brassica oleracea* var. *italica* Plenck cv. Green Comet. I. Effect of plant growth regulators. Ann. Bot. 58, 689-697.

LAZZERI P.A. & DUNWELL J.M. 1986b *In vitro* regeneration from seedling organs of *Brassica oleracea* var. *italica* cv. Green Comet. II. Effect of light conditions and explant size. Ann. Bot. 58, 699-710.

LEE N., WETZSTEIN H.Y. & SOMMER H.E. 1988 Quantum flux density effects on the anatomy and surface morphology of *in vitro*- and *in vivo*-developed sweetgum leaves. J. Am. Soc. Hortic. Sci. 113, 167-171.

LEGRAND B. 1972 Influence des conditions initiales d'éclairement sur le bourgeonnement et la polarité de tissus de feuilles d'endive cultivés *in vitro*. Compt. Rend. Acad. Sci. Paris 275D, 31-32.

LEGRAND B. 1974 Influence of the lighting conditions on the neoformation of shoots by leaf tissues of endive cultivated *in vitro* and on the peroxidase activity of the tissues. Compt. Rend. Acad. Sci. Paris 278D, 2425-2428.

LESHEM B., LILIEN-KIPNIS H. & STEINITZ B. 1982 The effect of light and of explant orientation on the regeneration and subsequent growth of bulblets on *Lilium longiflorum* Thunb. bulb-scale sections cultured *in vitro*. Sci. Hortic. 17, 129-136.

LETOUZÉ R. & BEAUCHESNE G. 1969 Action d'éclairements monochromatiques sur la rhizogenèse de tissus de topinambour. Compt. Rend. Acad. Sci. Paris 269, 1528-1531.

LETOUZÉ R. 1970 Maintien de la dominance apicale après décapitation d'une bouture de saule (*Salix babylonica* L.) en culture *in vitro*. Influence de la lumière bleue-violette. Compt. Rend. Acad. Sci. Paris. 271, 2309-2312.

LETOUZÉ R. 1974 The growth of the axillary bud of a willow cutting cultured *in vitro*: apical dominance and light quality. Physiol. Veg. 12, 397-412.

LETOUZÉ R. 1975 Growth of axillary buds of *Salix babylonica* and action of phenylalanine ammonia-lyase (PAL). Planta 123, 155-162.

LIN M.-L. & STABA E.J. 1961 Peppermint and spearmint tissue cultures. I. Callus formation and submerged culture. Lloydia 24, 139-145.

LISO R., INNOCENI A.M., BITONTI M.B. & ARRIGONI O. 1988 Ascorbic acid-induced progression of quiescent centre cells from G1 to S phase. New Phytol. 110, 469-471.

LITZ R.E. 1988 Somatic embryogenesis from cultured leaf explants of the tropical tree *Euphoria longan* Stend. J. Plant Physiol. 132, 190-193.

LONG C. (ed.) 1961 *Biochemists Handbook*. E. & F.N. Spon Ltd., London.

LORETI F., MULEO R. & MORINI S. 1991 Effect of light quality on growth of *in vitro* cultured organs and tissues. Comb. Proc. Int. Plant Prop. Soc. 1990 40, 615-623.

MACCHIA F., SCARAMUZZI F. & PORCELLI S. 1983 Organogenesis and propagation *in vitro* of F1 hybrid of *Solanum melongena* L. from vegetative segments. Acta Hortic. 131, 117-124.

MACKAY W.A. & NG T.J. 1988 Regeneration of *Cucumis melo* L. from cotyledon culture. HortScience 23, 753.

MACLEOD R.D. 1968 Changes in the mitotic cycle in lateral root meristems of *Vicia faba* following kinetin treatment. Chromosoma 24, 177-187.

MAENE L. & DEBERGH P. 1985 Liquid medium additions to established tissue cultures to improve elongation and rooting *in vivo*. Plant Cell Tissue Organ Cult. 5, 23-33.

MAHOTIERE S., HERNER R.C. & DENNIS F.G. 1976a Effects of applied growth substances on growth of shoot apices excised from onion in rest. J. Am. Soc. Hortic. Sci. 101, 211-213.

MAHOTIERE S., HERNER R.C. & DENNIS F.G. 1976b Effect of temperature on growth of shoot apices excised from onions in rest. HortScience 11, 154-155.

MANGAT, B.S., PELEKIS, M.K. AND CASSELLS, A.C. 1990 Changes in the starch content during organogenesis in *in vitro* cultured *Begonia rex* stem explants. Physiol. Plant 79, 267-274.

MARCOTRIGIANO M. & STIMART D.P. 1981 *In vitro* organogenesis and shoot proliferation of *Paulownia tomentosa* Steud. (Empress tree). HortScience 16, 405.

MARGARA J. & LEYDECKER M.-T. 1978 Différents types d'organogenèse observés chez le Colza, *Brassica napus* L. var. *oleifera* Metzg. Compt. Rend. Acad. Sci. Paris 287D, 17-20.

MARGARA J. 1969 Étude des facteurs de la néoformation de bourgeons en culture *in vitro* chez le chou-fleur (*Brassica oleracea* L. var. *botrytis*). Ann. Physiol. Veg. 11, 95-112.

MASCARENHAS A. F., GUPTA P. K., KULKARNI V. M., MEHTA U., IYER R. S., KHUSPE S. S. & JAGANNATHAN V. 1982 Propagation of trees by tissue culture. pp. 175-179 *in* Rao A.N. (ed.) 1982 (q.v.).

MATHUR J. 1991 Enhanced somatic embryogenesis in *Selinum candolii* DC. under a mineral oil overlay. Plant Cell Tissue Organ Cult. 27, 23-26.

McCLELLAND M.T. & SMITH M.A.L. 1990 Vessel type, closure and explant orientation influence *in vitro* performance of five woody species. HortScience 25, 797-800.

MCCREE K.J. 1972a The action spectrum, absorbance and quantum yield of photosynthesis in crop plants. Agric. Meteorol., 9, 191-216.

MCCREE K.J. 1972b Significance of enhancement for calculations based on the action spectrum for photosynthesis. Plant Physiol. 49, 704-706.

McGRANAHAN G.H., DRIVER J.A. & TULECKE W. 1987 Tissue culture of *Juglans*. pp. 261-271 *in* Bonga J. M.,& Durzan D. J., (eds.) *Cell and Tissue Culture in Forestry, Vol. 3. Case histories: Gymnosperms, Angiosperms and Palms.* Martinus-Nijhoff Publishers, Dordrecht, Boston, Lancaster. ISBN 90-247-3432-0.

MEYER M.M. Jr. 1982 *In vitro* propagation of *Rhododendron catawbiense* from flower buds. HortScience 17, 891-892.

MICHLER C.H. & LINEBERGER R.D. 1987 Effects of light on somatic embryo development and abscisic levels in carrot suspension cultures. Plant Cell Tissue Organ Cult. 11, 189-207.

MILLER C.O. 1956 Similarity of some kinetin and red light effects. Plant Physiol. 31, 318-319.

MILLER C.O. 1958 The relationship of the kinetin and red light promotions of lettuce seed germination. Plant Physiol. 33, 115-117.

MILLER L.R. & MURASHIGE T. 1976 Tissue culture propagation of tropical foliage plants. In Vitro 12, 797-813.

MISSON J.P., COUMANS M., GIOT-WIRGOT P. & GASPAR TH. 1982 Inductions de bourgeons adventifs sur bougeons de *Picea pungens* en culture *in vitro*. Z. Pflanzenphysiol. 107, 161-167.

MOHR H. 1987 Regulation from without: Darkness and light. Ann. Bot. 60, (Suppl. 4) 139-155.

MOK M.C. & MOK D.W. 1977 Genotypic responses to auxins in tissue culture of *Phaseolus*. Physiol. Plant. 40, 261-264

MOK M.C., MOK D.W.S., ARMSTRONG D.J., SHUDO K., ISOGAI Y. & OKAMOTO T. 1982 Cytokinin activity of N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (thiadiazuron). Phytochemistry 21, 1509-1511.

MOLNAR G.Y. 1987 A new patented method for mass propagation of shoot cultures. Acta Hortic. 212, 125-130.

MORRIS S.C., GRAHAM D. & LEE T.H. 1979 Phytochrome control of chlorophyll synthesis in potato tubers. Plant Sci. Lett. 17, 13-19.

MUKUND S., ROBACKER C. & SHEWFELT R. 1988 Callus and suspension cultures of tomato fruits for postharvest studies. HortScience 23, 754.

MURASHIGE T. & SKOOG F. 1962 A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15, 473-497.

MURASHIGE T. 1974 Plant propagation through tissue cultures. Annu. Rev. Plant Physiol. 25, 135-166.

MURASHIGE T. 1978 Principles of rapid propagation. pp. 14-24 in Hughes K.W. *et al,*(eds.) 1978 (q.v.).

NAIR S., GUPTA P.K. & MASCARENHAS A.F. 1983 Haploid plants from *in vitro* anther culture of *Annona squamosa* Linn. Plant Cell Rep. 2, 198-200.

NARASIMHULU S.B. & CHOPRA V.L. 1988 Species specific shoot regeneration response of cotyledonary explants of Brassicas. Plant Cell Rep. 7, 104-106.

NEGRUTIU I. & JACOBS M. 1978 Factors which enhance *in vitro* morphogenesis of *Arabidopsis thaliana*. Z. Pflanzenphysiol. 90, 423-430.

NEHRA N.S., STUSHNOFF C. & KARTHA K.K. 1988 Direct shoot regeneration from leaf discs of strawberry. HortScience 23, 808.

NEHRA N.S. & STUSHNOFF C. 1988 Regeneration of plants from immature leaf-derived callus of strawberry. HortScience 23, 756.

NESIUS K.K. & FLETCHER J.S. 1975 Contribution of non-autotrophic carbon dioxide to protein synthesis in suspension cultures of Paul's Scarlet rose. Plant Physiol. 55, 643-645.

NEUMANN K.H. 1968 Untersuchungen uber Beziehungen zwischen Zellteilung und Morphogenese bei Gewebekulturen von *Daucus carota*. II. Respiration von Gewebekulturen in Abhangigkeit von der Zellteilungintensitat. Physiol. Plant. 21, 519-524.

NICKELL L.G. & BURKHOLDER P.R. 1950 Atypical growth of plants. II. Growth *in vitro* of virus tumors of *Rumex* in relation to temperature, pH and various sources of nitrogen, carbon and sulfur. Am. J. Bot. 37, 538-547.

NIJKAMP H.J.J., VAN DER PLAS I.H.W. & VAN AARTRIJK J. (eds.) 1990 *Progress in Plant Cellular and Molecular Biology.* Proc. VIIth Int. Cong. on Plant Tissue and Cell Culture. Amsterdam, The Netherlands. 24-29 June 1990. Kluwer Academic Publishers, Dortrecht, Netherlands.

NITSCH J.P., ASHIRA T., ROSSINI L.M.E. & NITSCH C. 1970 Bases physiologique de la production de chair de pomme et de poire *in vitro*. Bull. Soc. Bot. Fr. 117, 479-492.

NORSTOG K. & KLEIN R.M. 1972 Development of cultured barley embryos. II. Precocious germination and dormancy. Can. J. Bot. 50, 1887-1894.

NORTON C.R. & NORTON M.E. 1986 Light quality and shoot proliferation in micropropagated *Prunus*, *Spiraea* and *Rhododendron*. p. 434 *in* Somers *et al.* (eds.) 1986 (q.v.).

NORTON C.R. & NORTON M.E. 1988a Induced dormancy of *Spiraea* cultured *in vitro* by low temperature and anoxic stresses. HortScience 23, 760.

NORTON C.R. & NORTON M.E. 1988b Manipulation of differentiation in *Spiraea in vitro*. Acta Hortic. 226, 191-194.

NORTON C.R. 1988 Metabolic and non-metabolic gas treatments to induce shoot proliferation in woody ornamental plants *in vitro*. Acta Hortic. 227, 302-304.

NORTON C.R., NORTON M.E., HERRINGTON T. & PHILLIPS D. 1988 Light quality and light pipe in the micropropagation of woody ornamental plants. Acta Hortic. 226, 413-416.

OBATA-SASAMOTO H., BEAUDOIN-EAGAN L. & THORPE T.A. 1984 C-metabolism during callus induction in tobacco. Plant Cell Tissue Organ Cult. 3, 291-299.

OBER E.S. & SHARP R.E. 2003. Electrophysical responses of maize roots to low water potentials: relationship to growth and ABA accumulation. J. Exp. Bot. 54, 813-824.

ORCHARD J.E., COLLIN H.A. & HARDWICK K. 1979 Culture of shoot apices of *Theobroma cacao*. Physiol. Plant. 47, 207-210.

OWEN J.H., LAYBOURN-PARRY J.E.M. & WELLBURN A.R. 1987 Influences of light quality, growth regulators and inhibitors of protein synthesis on respiration of protoplasts from meristematic cells in barley seedlings. Ann. Bot. 59, 99-105.

PAFFEN A.M.G., AGUETTAZ P., DELVALLEE I., DE KLERK G.J. & BOGERS R.J. 1990 The development of dormancy in lily bulblets generated *in vitro*. Acta Hortic. 266, 51-66.

PENCE V.C., HASEGAWA P.M. & JANICK J. 1980 Initiation and development of asexual embryos of *Theobroma cacao in vitro*. Z. Pflanzenphysiol. 98, 1-14.

PHILLIPS G.C. & HUBSTENBERGER J.F. 1985 Organogenesis in pepper tissue cultures. Plant Cell Tissue Organ Cult. 4, 261-269.

PIERIK R.L.M., STEEGMANS H.H.M. & VAN DER MEYS J.A.J. 1974b Plantlet formation in callus tissues of *Anthurium andraeanum* Lind. Scientia Hort. 2, 193-198.

PIERIK R.L.M. & STEEGMANS H.H.M. 1975 *Freesia* plantlets from flower-buds cultivated *in vitro*. Neth. J. Agric. Sci. 23, 334-337.

PIERIK R.L.M., STEEGMANS H.H.M. & MOLENBERG H. 1986 Vegetative propagation of *Syringa vulgaris* L. *in vitro*. p.434 *in* Somers *et al*. (eds.). 1986 (q.v.)

PIERIK R.L.M. 1987 Plant Cell Culture Technology - a book review. Sci. Hortic. 33, 308-309.

PIERIK R.L.M. 1988 *In vitro* culture of higher plants as a tool in the propagation of horticultural crops. Acta Hortic. 226, 25-40.

PIERIK R.L.M., STEEGMANS H.H.M., ELIAS A.A., STIEKEMA O.T.J. & VAN DER VELDE A.J. 1988a Vegetative propagation of *Syringa vulgaris* L. *in vitro*. Acta Hortic. 226, 195-204.

PIERIK R.L.M., VAN VOORST A., BOOY G., VAN ACKER C.A.M., LELIVELT C.L.C. & DE WIT J.C. 1988b Vegetative propagation of *Alstroemeria* hybrids *in vitro*. Acta Hortic. 226, 81-89.

PILLAI S.K. & HILDEBRANDT A.C. 1969 Induced differentiation of geranium plants from undifferentiated callus *in vitro*. Am. J. Bot. 56, 52-58.

PLUMB-DHINDSA P.L., DHINDSA R.S. & THORPE T.A. 1979 Non-autotrophic CO_2 fixation during shoot formation in tobacco callus. J. Exp. Bot. 30, 759-767.

POLEVAJA V.S. 1967 The effect of light of various spectral compositions on some aspects of auxin metabolism in cultures of isolated carrot tissues. Fiziol. Rast. 14, 582-591.

POWELL W. & UHRIG H. 1987 Anther culture of *Solanum* gemotypes. Plant Cell Tissue Organ Cult. 11, 13-24.

PREIL W., FLOREK P., WIX U. & BECK A. 1988 Towards mass propagation by use of bioreactors. Acta Hortic. 226, 99-106.

PUA E.-C., CHONG C. & ROUSSELLE G.L. 1983 *In vitro* propagation of Ottawa 3 apple rootstock. Can. J. Plant Sci. 63, 183-188.

RADOJEVIĆ L. 1979 Somatic embryos and plantlets from callus cultures of *Paulownia tomentosa* Steud. Z Pflanzenphysiol. 57-62.

RAHMAN M.A. & BLAKE J. 1988 Factors affecting *in vitro* proliferation and rooting of shoots of jack fruit (*Artocarpus heterophyllus* Lam.). Plant Cell Tissue Organ Cult. 13, 179-187.

RAO P.S. & NARAYANASWAMI S. 1972 Investigations in callus cultures of *Tylophora indica*. Physiol. Plant. 27, 271-276.

RAO A.N. (ed.) 1982 *Tissue Culture of Economically Important Plants*. Proc. Int. Symp., Singapore, 1981. COSTED and ANBS Publications.

READ P. & ECONOMOU A.S. 1983 Supplemental lighting in the propagation of deciduous azaleas. Comb. Proc. Int. Plant Prop. Soc. 1982 32, 639-645.

REINERT J., BAJAJ Y.P.S. & ZBELL B. 1977 Aspects of organization - organogenesis, embryogenesis, cytodifferentiation. pp. 389-427 in Street H.E. (ed.) 1977a (q.v.).

REYNOLDS J.F., BIEBER N.E. & SUN E.L. 1982 Environmental, genotype and pretreatment influences on regeneration of tomato *in vitro*. In Vitro 18: 318

RICHTER G. & WESSEL K. 1985 Red light inhibits blue light-induced chloroplast development in cultured plant cells at the mRNA level. Plant Mol. Biol. 5, 175-182.

RICHTER G., DANNHAUER I., KALDENHOFF R. & OTTERSBACH N. 1990 Blue-light effects on gene expression in plant cell cultures. pp.366-372 *in* Nijkamp *et al*. (eds.) 1990 (q.v.).

RIGHETTI B., MAGNANINI E. & MACCAFERRI M. 1988 Ethylene and other volatile substances produced by *in vitro*-cultured *Prunus avium*. Acta Hortic. 227, 402-404.

RODRGUEZ R., DIAZ-SALA C., CUOZZO L. & ANCORA G. 1991 Pear *in vitro* propagation using a double-phase culture system. HortScience 26, 62-64.

ROTHWELL K. & WRIGHT S.T.C. 1967 Phytokinin activity in some new 6-substituted purines. Proc. Roy. Soc. 167B, 202-223.

RUGINI E., TARINI P. & ROSSODIVITA M.E. 1987 Control of shoot vitrification of almond and olive grown *in vitro*. Acta Hortic. 212, 177-183.

RUTE T.N., BUTENKO R.G. & MAURINYA Kh.A. 1978 Effect of growth conditions on morphogenesis of apical meristem tissues from cucumber plants cultivated *in vitro*. Soviet Plant Physiol. 25, 432-438.

SAGAWA Y. & SHOJI T. 1967 Clonal propagation of *Dendrobiums* through shoot meristem culture. Am. Orchid Soc. Bull. 36, 856-859.

SAGLIO P.H., RANCILLAC M., BRUZAN F. & PRADET A. 1984 Critical oxygen pressure for growth and respiration of excised and intact roots. Plant Physiol. 76, 151-154.

SAID A.G.E. & MURASHIGE T. 1979 Continuous cultures of tomato and citron roots *in vitro*. In Vitro 15, 593-602.

SAMARTIN A. 1989 A comparative study of effects of nutrient media and cultural conditions on shoot multiplication of *in vitro* cultures of *Camellia japonica* explants. J. Hortic. Sci. 64, 73-79.

SANGWAN-NORREEL B.S. 1977 Androgenic stimulating factors in the anther and isolated pollen grain culture of *Datura innoxia* Mill. J. Exp. Bot. 28, 843-852.

SANTAMARIA, J.M., MURPHY, K.P., LEIFERT, C. & LUMSDEN, P.J. 2000 Ventilation of culture vessels. II. Increased water movement rather than reduced concentrations of ethylene and CO_2 is responsible for improved growth and development of *Delphinium in vitro*. J. Hortic. Sci. Biotech. 75, 320-327.

SATTER R.L. & GALSTON A.W. 1976 The physiological functions of phytochrome. pp. 680-735 *in* Goodwin T.W. (ed.)

The Chemistry & Biochemistry of Plant Pigments. Academic Press, London, New York, San Francisco.

SCHAFER E., BRUNS B., FROHNMEYER H., HAALBROCK K., HARTER K., MERKLE T & OHL S. 1990 Phytochrome in plant cell cultures. pp. 355-365 *in* Nijkamp *et al.* (eds.) 1990 (q.v.).

SCHEFFER F. & SCHACHTSCHABEL P. 1998 *Lehrbuch der Bodenkunde* 14th edition, Enke Verlag, Stuttgart, 494 p.

SCHNER S. & REINHARD E. 1982 Clonal multiplication of *Digitalis lanata* by meristem culture. Planta Med. 45, 155.

SCHNER S. & REINHARD E. 1986 Long-term cultivation of *Digitalis lanata* clones propagated *in vitro*: cardenolide content of the regenerated plants. Planta Med. 52, 478-481.

SCULLY R.M. Jr. 1967 Aspects of meristem culture in *Cattleya alliance*. Am. Orchid Soc. Bull. 36, 103-108.

SEABROOK J.E.A. 1987 Changing the growth and morphology of potato plantlets *in vitro* by varying the illumination source. Acta Hortic. 212, 401-410.

SEIBERT M., WETHERBEE P. & JOB D. 1975 The effects of light intensity and spectral quality on growth and shoot initiation in tobacco callus. Plant Physiol. 56, 130-139.

SENARATNA T., MCKERSIE B.D. & BOWLEY S.R. 1989 Desiccation tolerance of alfalfa (*Medicago sativa* L.) somatic embryos - influence of abscisic-acid, stress pretreatments and drying rates . Plant Sci. 65, 253-260.

SETHI U., BASU A. & GUHA-MUKHERJEE S. 1988 Control of cell proliferation and differentiation by regulating polyamine biosynthesis in cultures of *Brassica* and its correlation with glyoxalase-I activity. Plant Sci. 50, 167-175.

SHARP R.E. 2002 Interaction with ethylene: changing views on the role of abscisic acid in root and shoot growth responses to water stress. Plant Cell Environ. 25, 211-222.

SHARROCK R.A. & QUAIL P.H. 1989 Novel phytochrome sequences in *Arabidopsis thaliana*: structure, evolution and differential expression of a plant regulatory photoreceptor family. Gene. Dev. 3, 1745-1757.

SHIMADA N., TANAKA F. & KOZAI T. 1988 Effects of low O_2 concentration on net photosynthesis of C3 plantlets *in vitro*. Acta Hortic. 230, 171-175.

SHORT K.C., WARBURTON J. & ROBERT A.V. 1987 *In vitro* hardening of cultured cauliflower and chrysanthemum plantlets to humidity. Acta Hortic. 212, 329-334.

SIEGEL S.M. & PORTO F. 1961 Oxidants, antioxidants and growth regulation. pp. 341-353 *in Plant Growth Regulation*. Iowa State University Press, Ames, Iowa.

SIMMONDS J.A. & CUMMING B.G. 1976 Propagation of *Lilium* hybrids. II. Production of plantlets from bulb scale callus cultures for increased propagation rates. Sci. Hortic. 5, 161-170.

SKELLERN G.G. 1989 Thiocarbamides. pp. 49-89 *in* Damani L.A. (ed.) 1989 *Sulphur-containing Drugs and Related Organic Compounds: Chemistry, Biochemistry and Toxicology.* Vol. 1. Part B. *Metabolism of Sulphur Functional Groups*. Ellis Horwood Ltd. Chichester.

SMITH H. & KENDRICK R.E. 1976 The structure and properties of phytochrome. pp. 377-424 *in* Goodwin T.W. (ed.) *The Chemistry & Biochemistry of Plant Pigments.* Academic Press, London, New York, San Francisco.

SOFFER H. & BURGER D.W. 1988 Studies on plant propagation using the aero-hydroponic method. Acta Hortic. 230, 261-269.

SOMERS D.A., GEGENBACH B.G., BIESBOER D.D., HACKETT W.P. & GREEN C.E. (eds.) 1986 Abstracts VI Int. Cong. Plant Tissue and Cell Culture. Internat. Assoc. Plant Tiss. Cult. Minneapolis, Minn.

SOPORY S.K. & MAHESHWARI S.C. 1976 Development of pollen embryoids in anther cultures of *Datura innoxia* I. General observations and effects of physical factors. J. Exp. Bot. 27, 49-57.

STANDARDI A. & MICHELI M. 1988 Control of vitrification in proliferating shoots of M-26. Acta Hortic. 227, 425-427.

START N.G. & CUMMING B.G. 1976 *In vitro* propagation of *Saintpaulia ionantha* Wendl. HortScience 11, 204-206.

STETLER D.A. & LAETSCH W.M. 1968 Prolamellar body-like structures in tobacco tissue cultured on a medium lacking kinetin. Am. J. Bot. 55, 709.

STICKLAND R.G. & SUNDERLAND N. 1972a Production of anthocyanins, flavonols and chlorogenic acids by cultured callus tissues of *Haplopappus gracilis*. Ann. Bot. 36, 443-457.

STICKLAND R.G. & SUNDERLAND N. 1972b Photocontrol of growth, and of anthocyanin and chlorogenic acid production in cultured callus tissues of *Happlopappus gracilis*. Ann. Bot. 36, 671-685.

STIMART D.P. & ASCHER P.D. 1978 Foliar emergence and growth of *in vitro* generated bulblets from bulb scale explants of *Lilium longiflorum*. HortScience 13, 356.

STIMART D.P. 1986 Commercial micropropagation of florist flower crops. pp. 301-315 *in* Zimmerman R.H., Griesbach R.J., Hammerschlag F.A. and Lawson R.H. (eds.) *Tissue Cultures as a Plant Production System for Horticultural Crops*. Martinus Nijhoff, Dordrecht, Boston, Lancaster. ISBN 90-247-3378-2.

STIMART D.P., ASCHER P.D. & WILKINS H.F. 1983 Axis elongation from tissue cultured generated bulblets of *Lilium longiflorum* Thunb. J. Am. Soc. Hortic. Sci. 108, 99-101.

STONIER T., HUDEK J., VANDE-STOUWE R. & YANG H.-M. 1970 Studies of auxin protectors. VIII. Evidence that auxin protectors act as cellular poisers. Physiol. Plant. 23, 775-783.

STRAUB P.F., DECKER D.M. & GALLAGHER J.L. 1988 Tissue culture and long-term regeneration of *Phragmites australis* (Cav.) Trin. ex Steud. Plant Cell Tissue Organ Cult. 15, 73-78.

STREET H.E. 1957 Excised root culture. Biol. Rev. 32, 117-155.

STREET H.E. 1969 Growth in organised and unorganised systems - knowledge gained by culture of organs and tissue explants. pp. 3-224 *in* Steward F. C. (ed.) 1969. *Plant Physiology - a Treatise. 5B*. Academic Press, New York.

STREET H.E. (ed.) 1977a *Plant Tissue and Cell Culture*. Bot. Monographs Vol. 11 Blackwell Scientific Publications. Oxford, London.

STREET H.E. 1977b Laboratory organisation pp. 11-30 *in* Street H.E. (ed.) 1977a (q.v.).

STREET H.E. 1977c Cell (suspension) cultures - techniques. pp. 61-102 *in* Street H.E. (ed.) 1977a (q.v.).

STUART D.A., STRICKLAND S.G. & WALKER K.A. 1987 Bioreactor production of alfalfa somatic embryos. HortScience 22, 800-803.

STUART R. & STREET H.E. 1971 Studies on the growth in culture of plant cells. J. Exp. Bot. 22, 96-106.

SUNDERLAND N. & ROBERTS M. 1977 New approach to pollen culture. Nature 270, 236-238.

SUNDQVIST C., BJORN L.O. & VIRGIN H.I. 1980. Factors in chloroplast differentiation. pp. 201-224 *in* Reinert J. (ed.), *Results and problems in cell differentiation* vol. 10, *Chloro-plasts*. Springer-Verlag, Berlin, Heidelberg, New York

SUTTER E. & LANGHANS R.W. 1978 Tissue culture propagation of *Anemone coronaria*. HortScience 13, 348.

TAKAYAMA S. & MISAWA M. 1980 Differentiation in *Lilium* bulb scales *in vitro*. Effects of activated charcoal, physiological age of bulbs and sucrose concentration on differentiation and scale leaf formation *in vitro*. Physiol. Plant. 48, 121-125.

TAKAYAMA S. & MISAWA M. 1981 Mass propagation of *Begonia hiemalis* plantlets by shake culture. Plant Cell Physiol. 22, 461-467.

TAKAYAMA S. & MISAWA M. 1982 Mass propagation of ornamental plants through tissue culture. pp. 681-682 *in* Fujiwara (ed.) 1982 (q.v.).

TAKAYAMA S. & MISAWA M. 1983 A scheme for mass propagation of *Lilium in vitro*. Sci. Hortic. 18, 353-362.

TAKAYAMA S., MISAWA M., TAKASHIGE Y., TSUMORI H. & OHKAWA K. 1982 Cultivation of *in vitro*-propagated *Lilium* bulbs in soil. J. Am. Soc. Hortic. Sci. 107, 830-834.

TANAKA M., JINNO K., GOI M. & HIGASHIURA T. 1988 The use of disposable fluorocarbon polymer film culture vessel in micropropagation. Acta Hortic. 230, 73-80.

TANDEAU DE MARSAC N. & PEAUD-LENOEL C. 1972a Cultures photosynthétique de lignées clonales de cellules de tabac. Compt. Rend. Acad. Sci. Paris 274D, 1800-1802.

TANDEAU DE MARSAC N. & PEAUD-LENOEL C. 1972b Exchanges d'oxygène et assimilation de gaz carbonique de tissus de tabac en culture photosynthétique. Compt. Rend. Acad. Sci. Paris 274D, 2310-2313.

TEPFER D.A. & BONNETT H.T. 1972 The role of phytochrome in the geotropic behaviour of roots of *Convolvulus arvensis*. Planta 106, 311-324.

THOMAS D. DES S. & MURASHIGE T. 1979a Volatile emissions of plant tissue cultures. I. Identification of the major components. In Vitro 15, 654-658.

THOMAS D. DES S. & MURASHIGE T. 1979b Volatile emissions of plant tissue cultures. II. Effects of the auxin 2,4-D on production of volatiles in callus cultures. In Vitro 15, 659-663.

THOMPSON J.E., LEGGE R.L. & BARBER R.F. 1987 The role of free- radicals in senescence and wounding. New Phytol. 105, 317-344.

THORPE T.A. & LAISHLEY E.J. 1973 Glucose oxidation during shoot initiation in tobacco callus cultures. J. Exp. Bot. 24, 1082-1089.

THORPE T.A. 1980 Organogenesis *in vitro*: structural, physiological, and biochemical aspects. pp. 71-111 *in* Vasil I.K. (ed.) *Perspectives in Plant Cell and Tissue Culture*. Int. Rev. Cytology, Suppl. 11A, Academic Press.

TRAN THANH VAN K. 1977 Regulation of morphogenesis. pp. 367-385 *in* Barz *et al.* (eds.) 1977 (q.v.).

TRAN THANH VAN K. 1980 Control of morphogenesis by inherent and exogenously applied factors in thin cell layers. pp. 175-194 *in* Vasil I.K. (ed.) *Perspectives in Plant Cell and Tissue Culture*. Int. Rev. Cytology, Suppl. 11, Academic Press.

TRAN THANH VAN K., YILMAZ-LENTZ D. & TRINH T.H. 1987 *In vitro* control of morphogenesis in conifers. pp. 168-182 in Bonga and Durzan (eds.) 1987 (q.v.).

TRINH T.H. & TRAN THANH VAN K. 1981 *In vitro* flower formation from diploid and haploid thin layer of epidermal and subepidermal cells of *N. tabacum* L. and of *N. plumbaginifolia* Viv. Z. Pflanzenphysiol. 101, 1-8.

TUCKER D.J. & MANSFIELD T.A. 1972 Effects of light quality on apical dominance in *Xanthium strumarium* and the associated changes in endogenous levels of abscisic acid and cytokinins. Planta 102, 140-151.

TUCKER D.J. 1976 Effects of far-red light on the hormonal control of side shoot growth in the tomato. Ann. Bot. 40, 1033-1042.

UEDA H. & TORIKATA H. 1972 Effects of light and culture medium on adventitious root formation by Cymbidiums in aseptic culture. Am. Orchid Soc. Bull. 41, 322-327.

VAN DER PLAS L.H.W. & WAGNER M.J. 1986 Effect of oxygen on growth and respiration of potato tuber callus. Plant Cell Tissue Organ Cult. 7, 217-225.

VAN HUYLENBROECK J.M., PIQUERAS A. & DEBERGH P.C. 1998 Photosynthesis and carbon metabolism in leaves formed prior and during *ex vitro* acclimatization of micropropagated plants. Plant Sci. 134, 21-30.

VANDERHOVEN C. & ZYRD J.P. 1978 Changes in malate content and enzymes involved in dark CO_2 fixation during growth of *Acer pseudoplatanus* cells in suspension culture. Physiol. Plant. 43, 93-103.

VANDERSCHAEGHE A.M. & DEBERGH P.C. 1987 Technical aspects of the control of the relative humidity in tissue culture containers. pp. 68-76 *in* Ducaté *et al.* (eds.) 1987 (q.v.).

VANDERSCHAEGHE A.M. & DEBERGH P.C. 1988 Automation of tissue culture manipulations in the final stages. Acta Hortic. 227, 399-401.

VERMEER E. & EVERS P. 1987 A new technique to reduce labour by refreshing liquid medium automatically with a new container. pp. 264-266 *in* Ducaté *et al.* (eds.) 1987 (q.v.).

VERSLUES P.E., OBER E.S & SHARP R.E. 1998. Root grwoth and oxygen relations at low water potentials. Impact of oxygen availability in polyethylene glycol solutions. Plant Physiol. 116, 1403-1412.

VISEUR J. & LIEVENS C. 1987 *In vitro* propagation and regeneration of plants from calluses of *Begonia tuberhybrida*. Acta Hortic. 212, 705-709.

VISEUR J. 1987 Micropropagation of pear, *Pyrus communis* L., in a double-phase culture medium. Acta Hortic. 212, 117-124.

VON ARNOLD S. & ERIKSSON T. 1979 Bud induction on isolated needles of Norway spruce (*Picea abies* L. Karst) grown *in vitro*. Plant Sci. Lett. 15, 363-372.

WALKEY D.G.A. & COOPER J. 1976 Growth of *Stellaria media*, *Capsella bursa-pastoris* and *Senecio vulgaris* plantlets from cultured meristem tips. Plant Sci. Lett. 7, 179-186.

WALKEY D.G.A. & WOOLFITT J.M.G. 1970 Rapid clonal multiplication of cauliflower by shake culture. J. Hortic. Sci. 45, 203-206.

WAN ABDUL R., GHANDIMATHI H., ROHANI O. & PARANJOTHY K. 1981 Recent developments in tissue culture of *Hevea*. International Rubber Res. Development Board Symposium, Haadyai, 1981.

WANG P.-J. & HU C.-H. 1982 *In vitro* mass tuberization and virus-free seed potato production in Taiwan. Am. Pot. J. 59, 33-37.

WARD H.B. & VANCE B.D. 1968 Effects of monochromatic radiations on growth of *Pelargonium* callus tissue. J. Exp. Bot. 19, 119-124.

WEGG S.M. & TOWNSLEY P.M. 1983 Ascorbic acid in cultured tissue of Briar Rose, *Rosa rugosa* Thunb. Plant Cell Rep. 2, 78-81.

WEIS J.S. & JAFFE M.J. 1969 Photoenhancement by blue light of organogenesis in tobacco pith cultures. Physiol. Plant. 22, 171-176.

WELANDER M. 1987 *In vitro* culture of raspberry (*Rubus idaeus*) for mass propagation and virus elimination. Acta Hortic. 212, p. 610.

WELANDER M. 1988 Plant regeneration from leaf and stem segments of shoots raised *in vitro* from mature apple trees. J. Plant Physiol. 132, 738-744.

WELANDER T. 1978 *In vitro* propagation of clones from different cultivars of *Begonia hiemalis* sexual hybrid. Swed. J. Agric. Res. 8, 181-186.

WIMBER D.E. 1965 Additional observations on clonal multiplication of cymbidiums through culture of shoot meri-stems. Cymb. Soc. News 20, (4) 7-10.

YAE B.W., ZIMMERMAN R.H., FORDHAM I. & KO K.C. 1987 Influence of photoperiod, apical meristem and explant orientation on axillary shoot proliferation of apple cultivars *in vitro*. J. Am. Soc. Hortic. Sci. 112, 588-592.

YEOMAN M.M. & DAVIDSON A.W. 1971 Effect of light on cell division in developing callus cultures. Ann. Bot. 35, 1085-1100.

YEOMAN M.M. & STREET H.E. 1977 General cytology of cultured cells. pp. 137-176 *in* Street H.E. (ed.) 1977a (q.v.).

ZELITCH I. 1978 Improving the efficiency of photosynthesis. Science 188, 626-633.

ZIMMERMAN R.H. 1984 Chapter 14 – Apple. pp. 369-395 *in* Sharp W.R., Evans D.A., Ammirato P.V. and Yamada Y. (eds.) *Handbook of Plant Cell Culture. Vol. 2. Crop Species*. MacMillan Publishing Co., New York, London.

ZOBAYED S.M.A., AFREEN F. & KOZAI T. 2000. Quality biomass production via photoautotrophic micropropagation. Acta Hortic. 530, 377-386.

Chapter 13
The Anatomy and Morphology of Tissue Cultured Plants

1. INTRODUCTION

Tissue culture plants, unlike zygotic plants, originate from existing axillary buds and somatic cells through either organogenesis or somatic embryogenesis, depending on the explant type and culture conditions. In the organogenic pathway only one organ, a root or a shoot, is initiated from a unipolar meristem, while in the embryogenic pathway a bipolar structure develops with shoot and root meristems. Plants developed *via* organogenesis have mostly arisen from buds that form adventitious roots after an induction phase. The vascular connections at the root-to-shoot interface are of major importance for the function of the vascular system and the viability and survival of the plants after transplanting. Plants that develop from somatic embryos have a root and a shoot system, which under optimal conditions, usually develop normal vascular connections.

The morphogenetic expression of root, stem and leaves *in vitro* results from an interaction between extrinsic and intrinsic factors and from various components of the cultural condition. The structure and function of a regenerated plant depend on *in vitro* developmental signals that are related to the environmental condition in culture. As yet we do not fully understand the signals that permit or direct the developmental chain of reactions. Developmental changes follow a sequence of events and are coordinated in time and space, and therefore any interruption in the signal-transduction chain may result in a disturbed morphogenetic expression. Furthermore, the isolation and culture of an explant in a defined medium not only changes internal existing gradients, but also alters the sensitivity of the tissue to triggering signals and to induction of stress, which alter the physiological state of plant tissue due to anatomical and morphological malformation (Kevers *et al.,* 2004). This can shift meristematic activity to a pattern unlike the one initiated prior to these changes, or to the one that exists in the plant *in vivo* (Ziv, 1995; 1999).

The organization of the specialized organs and nature of the various tissues of cultured material, determine the survival potential of plants *ex vitro*. Most of the anatomical studies carried-out on *in vitro* plants cover three major aspects: the first, the initiation phases of meristems and their development into either roots or bud apices; the second, phases of somatic embryo development and the evolving plantlet; and the third, relates to anatomical and histological changes or to the deformation – the phenomenon of hyperhydricity ('vitrification') – in organs and plants *in vitro,* and in plants after they have been transplanted *ex vitro.* Hyperhydric (or 'vitrified') organs appear to be water-soaked and are brittle with an abnormal structure. Hyperhydric shoots are easily damaged by desiccation and survive very poorly when subcultured or transferred to an external environment.

The present chapter discusses briefly the anatomy of meristemoid and organ initiation, and covers mainly the whole plant, describing the anatomy and histology of roots, stems and leaves *in vitro*, in relation to their development *extra vitrum*.

2. ROOT DEVELOPMENT *IN VITRO*

Micropropagated plants, originating through either somatic embryogenesis or an organogenic pathway, require a fully developed root system prior to transplanting *ex vitro* to endure the external environmental stress. Rooting of shoots can take place *in vitro*, or after transplanting as part of the acclimatization process *ex vitro.*

Adventitious roots can be induced in different types of tissue depending on the cultural conditions and the parent organs. The ratio between auxin and cytokinins in the medium may determine the type of morphogenetic response occurring *in vitro*. Usually, elevated auxin levels are required to promote rooting, particularly during the hardening stage. In general, adventitious roots that develop in agar are thin, but may thicken in high auxin media or in the presence of growth retardants (Roberts *et al.,* 1992).

Adventitious root formation varies and depends on the genotype and on cultural methods (see Chapter 9). Generally, induction of roots is more difficult in woody species than in herbaceous species (Hackett, 1985). De Klerk *et al.,* (1995) distinguished between three main phases in the rooting process in microshoots of apple *(Malus domestica* Borkh) - dedifferentiation; induction; and morphological differentiation. There is a controversy regarding the

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general definition of the various phases in the rooting process. In some species (mungbean, poplar), when stem cuttings are taken, root initials are already present in the stems and are directly involved in the rooting morphological differentiation phase. Formation of root initials after an intermediate callus phase is obvious evidence for the occurrence of the three phases. An indirect rooting process through callus is usually associated with prolonged phases of dedifferentiation and re-differentiation (De Klerk *et al.,* 1999).

2.1. ANATOMICAL PHASES IN ROOT DEVELOPMENT

Anatomical time-course studies have revealed differences in the response and involvement of various cell layers and organelles in adventitious root formation. Root formation can be either direct - from the plant tissue, or indirect – following callus formation (Apter *et al.,* 1993; Harbage *et al.,* 1993; Welander and Pawlicki, 1993; Jasik and De Klerk, 1997; Pinker, 2000).

Root primordia initiation and organized root development occur before the emergence of the root tip from the stem (Esau, 1977). Adventitious roots are often induced in parenchyma cells (Lovell and White, 1986). Microcuttings of *Malus domestica* have been used to study root formation to observe developmental events leading to root formation (Zimmerman and Fordham, 1985; De Klerk *et al.,* 1995; Jasik and De Klerk, 1997). The duration and location of root development events between taxa and even between conventional and micropropagated material can vary (Lovell and White, 1986).

Anatomical studies of the stem bases of tissue cultured Asian jasmine (*Trachelospermum asiaticum*) revealed root primordia on the third day that appeared as groups of 25 to 75 cells containing darkly stained cytoplasm and nuclei. The cells originated external to the cambium in the phloem. Well-defined root primordia with xylem cells developed after a week and penetrated through the periderm (Apter *et al.,* 1993).

In microcuttings of "Gala" apples, adventitious root initiation was detectable within 24 hours in phloem parenchyma cells that became densely cytoplasmic, containing nuclei with prominent nucleoli. Meristematic activity in the phloem parenchyma continued and advanced into the cortex forming root primordia with distinct cell layers at the distal end. These conically shaped primordia formed a well-organized root tissue system. The initiation of cell division in a rooting medium took place in parenchyma cells of the phloem, cortex, cambial region, xylem, and pith tissue. However, only the divisions of the phloem parenchyma cells resulted in meristematic regions, leading to root primordia. Meristemoids were initiated in the phloem tissue around the ring of vascular bundles (Harbage *et al.,* 1993). Welander and Pawlicki (1993) found an increased cambial activity around the vascular bundles in apple rootstock Jork M9 stem discs. Cells with densely stained cytoplasm and enlarged nucleoli appeared between the vascular bundles, external to the phloem, forming meristemoids that later differentiated into root primordia. In some other studies of micropropagated woody species, the sites of root origin have been located in cells within or just external to the vascular cambium or adjacent to the cortex. Cells leading to root formation could have been phloem parenchyma or derivatives of cambial cells normally destined to phloem parenchyma formation (Hicks 1987; Iliev *et al.,* 2001; Ranjit *et al.,* 1988; Samartin *et al.,* 1986).

Light, and electron microscopy studies, of *in vitro* root formation in stem slices of apple cv. Jork 9 (*Malus domestica* Borkh.) microshoots, revealed starch grains in the cambial region, in the vicinity of the vascular tissue and the primary rays. The nuclei in the cells were activated, and the density of the cytoplasm and the number of cell organelles increased, while starch was broken down. Cambial cells begun to divide transversely, forming a continuous ring of isodiametric cells around the xylem, near the basal surface of the cutting. The cells that were rich in cytoplasm and organelles and lacked starch grains and a central vacuole, developed into root meristemoids in the primary rays. After emergence, the meristemoids were transformed into small, dome-like primordia and developed a typical root apex with root cap and root ground meristem. In stem slices, many cells participated in the early divisions, but only a few cells developed into root meristems (Jasik and De Klerk, 1997).

Histological studies of adventitious root formation in different aged cuttings of *Prunus kurilensis* and *Amelanchier lamarckii* revealed differences in rooting potential depending on the age of the shoots. The obvious differences in the vascular system, particularly the ray parenchyma, might be important for root induction. In easy-to-root *P. kurilensis*, the older stems formed more xylem and phloem vessels, with an epidermis and a phellem that later surrounded the stem. Starch grains were frequent in the pith and cortex in old stems. In difficult-to-root *A. lamarckii*, the stem vascular system developed very slowly and

the differences between stems were not obvious as in *P. kurilensis*. The extent of starch accumulation was lower than in *P. kurilensis*. With a prolonged subculture period some phloem cells collapsed. While root primordia formed in *P. kurilensis*, only primordia or meristemoids initials were formed in *A. lamarckii* (Pinker, 2000).

Soukup *et al.,* (2004) investigated the lignification of the root system in fast growing cherry (*Prunus avium*) and slow growing oak (*Quercus robur*) during acclimatization and found different lignification patterns in the primary root tissues of the two species. The most conspicuous difference was the appearance of thickenings in the cortical layers next to the endodermis in cherry roots that appeared after transfer to *extra vitrum* conditions. Later, when secondary tissues appeared, lignified secondary xylem constituted most of the tissues of both species. Thin walled cells and less suberised dermal tissues were found in cherry plants while multi-layered and well-developed cork tissue was observed in oak plants.

Considerable progress has been achieved in rooting research, and has indicated that rooting is a process consisting of distinct phases, with specific requirements at each phase (De Klerk *et al.,* 1999).

2.2. THE ANATOMY OF THE ROOT-TO-SHOOT INTERFACE

In woody species, roots that developed in agar-solidified media, had large hypertrophied cortical cells and lacked a secondary vascular system (McClelland *et al.,* 1990; Rogers and Smith, 1992). *In vitro* rhizogenesis in herbaceous plants is found to be easier than rooting in woody species. Smith *et al.* (1991, 1992) found that woody species often produced more roots per shoot, but the roots were underdeveloped, with limited secondary vascular cambium activity, which was resumed only after *extra vitrum* transplanting. The interaction of shoot quality and rooting potential (in particular, a poor vascular development at the root-to-shoot interface), has often been found to contribute to poor survival of plantlets during their acclimatization from tissue culture (Conner and Thomas, 1981; Pierik, 1987; Ziv, 1986; Pinker, 2000).

Smith *et al.,* (1991) reported that the vascular connections between new *in vitro* roots and the microcutting stems of maple, birch, and apple were often discontinuous, since the *in vitro* roots were often subtended by callus or emerged from callus. Some of the roots became blackened shortly after emergence, and failed to continue to elongate. Inspection of cross sections showed that the black color was restricted to a cuticular coating around the epidermal cells of the root. In plantlets of cauliflower (*Brassica oleracea* L), vascular connections between roots and shoots were found to be poorly developed and very narrow (Grout and Aston 1977).

By contrast, in some other plants, for example Asian jasmine (*Trachelospermum asiaticum*), apple (Hicks, 1987; Harbage *et al.,* 1993; Jasik and De Klerk, 1997), *Pinus sylvestris* (Gronroos and Von Arnold, 1985), and black and white spruce (Patel *et al.,*1986), vascular discontinuity between adventitious roots and parental shoots has not been detected. Early xylem cells with helical wall thickenings were evident at the root primordium base of Asian jasmine, and were connected to the secondary xylem tissue in the shoot system. Elongation of the emerging root and further xylem development within the adventitious roots and at the root-to-shoot interface continued for up to days 8 to 10. Afterward there was no evidence that xylem elements were not continued between the elongating primordia and the base of the primordia adjacent to the cambium (Apter *et al.,* 1993). Similarly, newly-developed root primordia or emerging roots on micropropagated shoots of apple (*Malus domestica* Borkh.), cv. Jork 9 (Jasik and De Klerk, 1997) and cv. Gala (Harbage *et al.,* 1993) were also found to have tracheid connections continuous with those of the shoot vascular tissue.

Auxin increased cambial activity and adventitious root formation on *Betula pendula* microcuttings. At the base of the roots, tracheid-like cells formed providing vascular connection between the root-shoot system (Iliev *et al.,* 2001).

The differentiation of the vascular tissue at the root-to-shoot interface needs to be further examined in relation to the function of the vascular tissue of plants regenerated *in vitro* and their subsequent survival *ex vitro*.

3. LEAF DEVELOPMENT AND STRUCTURE

Bud meristem development was observed by Bornman and Vogelmann (1984) to initiate from almost all the plant organs and tissues they isolated for *in vitro* establishment, but could be affected by various culture conditions at very early stages of leaf primordial development (Fig. 13.1). In most plants,

leaf primordia developed *in vitro,* contain all the various components of normal leaf tissue, but in many cases they are not fully developed and remain so for a long time after the culture period.

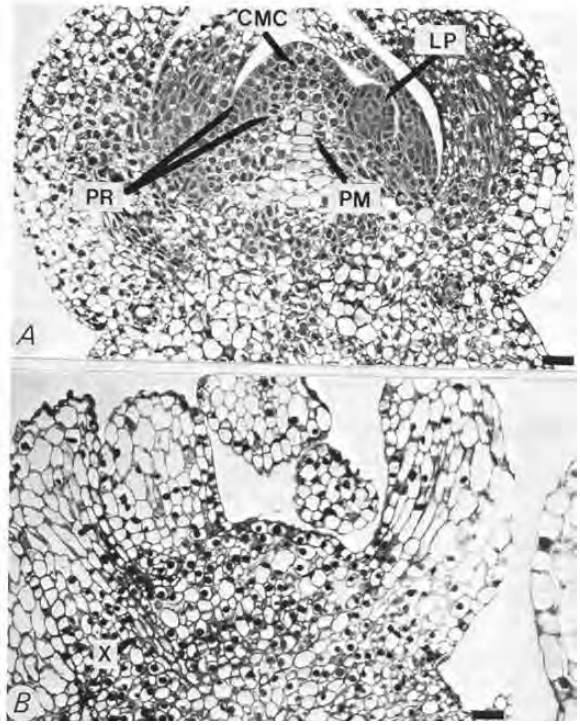

Fig. 13.1 Longitudinal section of a normal A and a hyperhydric B *Picea abies* bud showing the meristem and leaf primordial. CMC – Central mother cell, LP – leaf primordial, PM - pith meristem, PR – peripheral regions, X – xylem (Bar 50 µm) (Bornman and Vogelmann, 1984).

3.1. THE MESOPHYLL

Microscopically examined leaves of several tissue cultured plants have been observed to have distorted anatomy with a very thin palisade tissue. It often either consisted of only one layer of more rounded than elongated cells, or was absent altogether. Most of the mesophyll tissue consisted of spongy parenchyma with enlarged vacuolated cells, rich in intercellular spaces. In apple, the applets system was hyper-developed, increasing the volume of the tissue up to fivefold in comparison to normal leaves (Paques and Boxes, 1987; Ziv, 1991), whereas an increase in intercellular spaces within cultured carnation shoots was due to excess apoplectic water (Kevers and Gaspar, 1986). The cells in vitreous leaves were surrounded by very thin cell walls and contained a relatively thin cytoplasm, enclosing a large vacuole (Brainerd *et al.,* 1981; Leshem, 1983). Many of the chloroplasts of sweetgum lacked the normal organization into grana and stroma and the chloroplasts of *in vitro*-grown plants lacked starch granules and were flattened with disorganized grana. However, chloroplasts with well-organized grana developed in the leaves when the light level during culture was raised from 50 to either 155 or 315 µmol.m^{-2}.s^{-1} (Wetz-tein and Sommer, 1982, 1983; Lee *et al.,* 1985). Analysis of electron micrographs of palisade cells showed that the functional area was largest in chloroplasts of leaves subjected to blue light and smallest in those exposed to red light (Saebo *et al.,* 1995).

The hyperhydric leaves of a *Eucalyptus* hybrid had chloroplasts with small grana, an absence of starch grains and a higher density of endoplasmic reticulum in comparison to leaves in normal shoots (Fig. 13.2 A,B – Louro *et al.,* 1999). However, the chloroplasts of carnation shoots cultured by Ziv and Ariel (1994) in liquid medium, contained large starch grains. The hyperhydric leaves of carnation had large vacuolated mesophyll cells, showing a hypertrophy of the cells with larger intercellular spaces than in normal plants. In these leaves cuticular wax was absent, and chloroplasts presented abundant plastoglobuli (Olmos and Hellin, 1998). 'Compact' hyperhydric leaves of eggplant contained one layer of slightly differentiated palisade cells, whereas in 'lacunous' leaves an increase was observed in the length of the spongy parenchyma cells, which was linked to an increase in intercellular spaces (Picoli *et al.,* 2001).

3.2. EPIDERMIS AND CUTICULE STRUCTURE

In several species, leaf hyperhydricity has been associated with defective epidermal tissue. The cuticle thickness and chemical nature were different from field or greenhouse grown plants. In these plants, the crystalline structure of epicuticular waxes on the leaves of micropropagated plants was significantly different to that on normal plants. Epicuticular waxes were deposited thinly and their composition in vitreous and normal leaves was significantly different (Preece and Sutter, 1991). Faulty deposition of cuticular waxes on vitreous leaves of cabbage, cauliflower and carnation was manifested in both quantity and quality of the waxes and the cuticle lacked the characteristic structure of *in vivo* normal plants (Grout, 1975; Grout and Aston, 1978; Sutter and Langhans, 1982; Sutter, 1985; Ziv *et al.,* 1981, 1983). Epidermal cell areas were largest in plantlets subjected to blue light and smallest in those subjected to red light (Saebo *et al.,* 1995). In *Prunus*

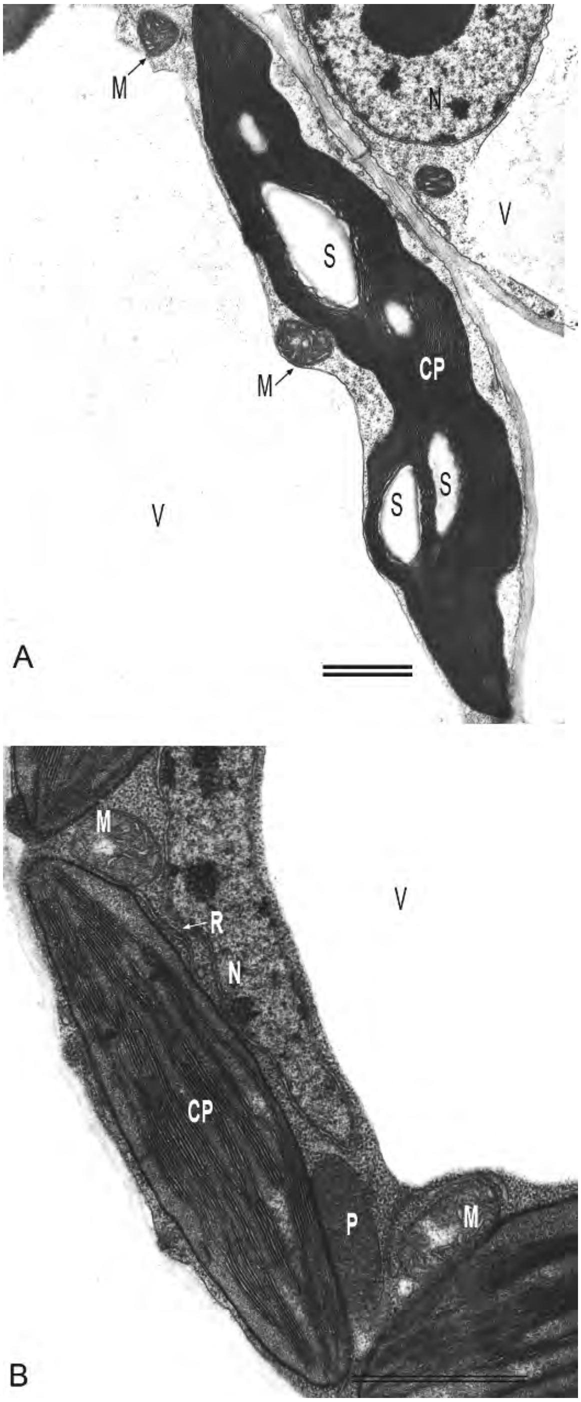

Fig. 13.2 Normal (A) and hyperhydric (B) palisade cells in *Eucalyptus*. CP – chloroplast, M – mitochondria N – nucleus, S - starch grains, V – vacuole, P– peroxisome, R – endoplasmic reticulum (Bar 1.2 μm) (Louro *et al*., 1999).

avium the cuticle in normal leaves exhibited striation, which was not observed in hyperhydric leaves (Franck *et al.*, 1997).

Leaves newly formed after transplanting develop increasing amounts of wax and more complex crystalline structures over time (Sutter and Langhans, 1982; Preece and Sutter, 1991) as has been shown with carnation (Sutter and Langhans, 1979), cauliflower (Grout and Aston, 1977), and cabbage (Sutter, 1988). The lack of epicuticular wax on these plants *in vitro* was due to the high humidity within the culture vessel. Sutter and Langhans (1982) produced cabbage plants with normal cuticle and structured wax by reducing the relative humidity *in vitro* to 35%. Wardle *et al.*, (1983) produced cauliflower plants with clearly higher amounts of epicuticular wax by reducing the relative humidity *in vitro* by using a desiccant.

Leaves of grape plantlets treated with PEG (polyethylene glycol) in the culture vessel, had a greater deposition of epicuticular wax than plants grown in the greenhouse or the controls. Stomatal index did not vary among treatments but differences in wax deposition, leaf epidermal cell configuration and modified leaf surface anatomy, were associated with increased *ex vitro* survival after 4 weeks in the greenhouse (Dami and Hughes, 1997). Leaves of cabbage plants grown *in vitro* never became as glaucous as the new leaves that formed after removal from culture (Grout, 1975). There were less filiform trichomes on the abaxial surfaces of raspberry leaves grown *in vitro* than in transplanted plants grown in the greenhouse (Donnelly and Vidaver, 1984).

3.3. THE STOMATAL APPARATUS

In several plant species cultured *in vitro,* the guard cells in the stomatal apparatus of the leaf epidermal tissue did not function properly and remained open in darkness, even under water stress conditions (Ziv, 1991). This was associated with changes in the structure of the stomata which were raised, rounded (instead of elliptical, as in normal leaves), and had damaged cell walls as can be seen in Fig.13.3 (Ziv and Ariel, 1994). Abnormally large stomata were observed in geranium and rose (Reuther, 1988), and have also been seen on the leaves of *Solanum laciniatum* (Conner and Conner, 1984), rose (Capellades *et al.,* 1990) and apple (Blanke and Belcher, 1989). The stomata of apple, carnation and cauliflower did not close in response to treatment with ABA, high CO_2, Ca^{++} or hypertonic solutions of mannitol or sucrose, due to faulty

deposition of cellulose (Brainerd *et al.,* 1981; Fuchigami *et al.,* 1981; Wardle *et al.,* 1979; Ziv *et al.,* 1987). Faulty orientation of microfibrils affects the stomatal aperture movement (Palevitz, 1981), which may explain the failure of vitreous guard cells to close.

Histochemical studies of guard cells from vitreous carnation leaves revealed lower levels of cutin, pectins and cellulose (Ziv and Ariel, 1992, 1994; Werker and Leshem, 1987; Koshuchowa *et al.,* 1988). Callose deposits have been reported in the guard cells of vitreous cherry and carnation leaves. The cell walls lacked the normal orientation of cellulose microfibrils typical of guard cells in greenhouse or field grown plants (Ziv *et al.,* 1987; Marin *et al.,* 1988; Ziv and Ariel, 1992, 1994). An increase in light level from 25 to 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a decrease in relative humidity from 100 to 75% resulted in the stomata of rose plants grown *in vitro* being similar to those on leaves formed in the greenhouse. However the stomata were depressed, more ellipsoid, and less abundant. Large starch grains in the chloroplasts of these rose plants appeared after transfer to 5% sucrose medium (Capellades *et al.,* 1991). In a similar study, no change was found in the stomatal morphology of sweet gum when only the light level was altered *in vitro* (Lee *et al.,* 1988). Stomata on leaves of raspberry plants removed from culture, and thus under a lower relative humidity than when *in vitro,* remained raised under low light and became sunken under a high light level (Donnely and Vidaver, 1984).

The failure of the cell walls to contract in hypertonic solution was shown to correlate with the abnormal orientation of cellulose microfibrils and the high deposition of callose in the malformed guard cells of vitreous carnation leaves (Ziv and Ariel, 1992, 1994).

Thus, hypolignification, microfibril disorientation and the deposition of callose instead of cellulose in the cell walls could have contributed to stomatal malfunction and the phenomenon of hyperhydricity.

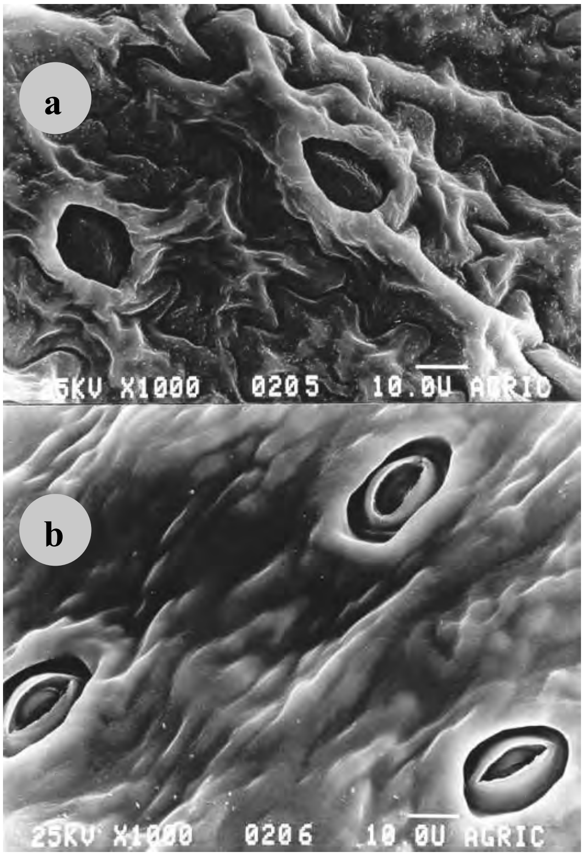

Fig. 13.3 Stomata from a normal (a) and a hyperhydric *Dianthus caryophyllus* leaf (b) (Ziv and Ariel, 1994).

4. HYPERHYDRICITY AND MALFORMATION IN CULTURED PLANTS

Morphogenesis is affected by continuous changes of various signals from the immediate cultural environment. Conditions *in vitro* foster a high degree of cell plasticity, which in many plant species can lead to developmental aberration. Herbaceous and woody plants propagated *in vitro* are frequently affected by the redundant presence of various factors that lead to metabolic and morphological derangements and resultant hyperhydricity (Ziv, 1991, 1999).

Chestnut (*Castanea sativa* Mill) hyperhydric shoots were produced during successive subcultures when MS mineral medium was used. Xylem and sclerenchyma tissues were less differentiated with hypertrophy in the cortical and pith parenchyma. Examination of transverse sections from various regions of the stems of these shoots showed that certain tissues were less differentiated than normal. In the basal region, the perivascular sclerenchyma ring was usually reduced to small isolated groups of the fibers exhibiting hardly any lignification, and in some specimens the sclerenchyma was almost absent. Likewise, xylem tissue was comparatively less differentiated (and hence poorly lignified). In the cortex, there was an increase in both the number and size of the cells, with a particularly marked hypertrophy in the deep layers of the cortical

parenchyma. Large intercellular spaces also tended to appear, lending the tissue a semi-disintegrated appearance. This greatly expanded cortex led to a larger than normal stem diameter. In addition, there was a basipetal gradient of lignification and development of xylem and sclerenchyma from the apical region, where there was no differentiated sclerenchyma ring and the xylem was reduced to lignified protoxylem and non-lignified metaxylem (Vieitez *et al.,* 1985). Stem elongation of hyperhydric melon and carnation plantlets was retarded. Cells became hypertrophied, and the intercellular spaces were much larger than in normal cells (Leshem *et al.,* 1988).

Compared to the leaves on normal shoots of *Eucalyptus grandis* x *Eucalyptus urophylla*, hyperhydric leaves on shoots with malformed stems possessed a reduced number of stomata on their adaxial surface and enlarged palisade cells. The hyperhydric stems showed chloroplasts with small grana, an absence of starch grains, and a higher density of rough endoplasmic reticulum at the ultrastructural level (Louro *et al.,* 1999).

The hyperhydricity in eggplant (*Solanum melongena* L.) plants was monitored by the induction of the ER-luminal resident protein BiP (Binding Protein), an endoplasmic reticulum-resident molecular chaperone. The accumulation of BiP in hyperhydric shoots was consistently higher than in normal shoots. In non-hyperhydric organs there were smaller and more organized cells, besides a more differentiated vascular system compared with their hyperhydric counterparts. Examination of transverse sections of hyperhydric stems revealed that they were larger in size and displayed cortical cells with large intercellular spaces and areas with thinner xylem and less lignified cell walls. Typically, hyperhydric cortex and pith displayed cells with irregular contours and hyperhydric stems displayed a significant increase in pith parenchyma area. Large intercellular spaces appeared in the cortex, and the expanded cortex and pith led to a larger stem diameter. A vascular cambium, differentiating lignified secondary xylem elements between the xylem and the phloem was observed. Therefore, hyperhydric eggplant stems displayed secondary vascular tissue that was less differentiated, resulting in a poorly lignified xylem tissue and a thinner vascular ring (Picoli *et al.,* 2001).

Iliev *et al.,* (2003) compared *in vitro* cultured normal and fasciated stems of *Betula pendula* Roth. Normal stems were rounded and had a well-developed cortex and vascular tissue, the concentric ring of the xylem consisting of 5-10 rows of xylem cells with well-developed secondary walls. By contrast, fasciated stems were elliptical or with irregular sharp edges on transverse sections. Their vascular tissues were less developed, or at a much earlier stage of differentiation than was normal and the differentiation of xylem was delayed. It was suggested that the appearance of fasciated stems might be caused by suppression of a gene under the influence of zeatin. The most striking difference between normal and fasciated stems was the shape of the vascular cylinder and the pattern of development of the vascular tissues.

4.1. MORPHOGENIC DIFFERENCES BETWEEN *IN VITRO* AND *EX VITRO* STEMS ROOTS AND LEAVES

The significant differences between plants *in vitro* and after transplanting *extra vitrum,* which are exhibited in the roots and leaves and to a lesser extent in the stems, are summarized in Tables 1 and 2.

Differences in morphogenetic development between *in vitro* and *ex vitro* stems and roots have been studied in raspberry, maple, birch, forsythia, apple, miniature rose and jasmine (Donnelly *et al.,* 1985; Smith *et al.,* 1991; Apter *et al.,* 1993). Stems of *in vitro* red raspberry plantlets were smaller in diameter than control plant stems. *In vitro* stems had little collenchyma in the cortex, very few sclerenchyma fibers and no thick walled pith cells. Control stems had a continuous cylinder of collenchyma in the cortex beneath the epidermis, a thick layer of sclerenchyma in the phloem of the stele and both thin and thick walled pith cells. *In vitro* roots of red raspberry plantlets were smaller in diameter, white or pinkish in color and had little periderm. The roots were covered with fine root hairs and had a delicate appearance, while *ex vitro* roots of the control plants were brown in color and had a multi-layered periderm. *In vitro* red raspberry transplants that had been grown under high light intensities, had a larger stem diameter and had developed better support tissue (Donnelly *et al.,* 1985). Cellular differentiation in excised *in vitro* pea roots began closer to the apex, the cortical and epidermal cells were shorter and the mitotic activity in the pericycle increased following exposure to salinity stress (Solomon *et al.,* 1986).

Table 13.1 Anatomical and histological differences between roots and stems of plants *in vitro* and after transplanting *ex vitro*

Plant Organ and Tissue	*In vitro*	*Ex vitro*	Species studied
ROOTS	Thin	Large root system	**Root and stem**: *Acer rubrum* L. *Amelanchier lamarckii* *Betula nigra* L. *Betula pendula* Roth *Brassica oleracea* L. *Castanea sativa* Mill *Forsythia x intermedia* Zab. *Malus domestica* Borkh *Pinus sylvestris* *Pisum sativum* *Prunus kurilensis* *Quercus robur* *Rosa chinensis minima* *Rubus idaeus* L. *Solanum melongena* L. *Trachelospermum asiaticum*
Epidermis	Uniseriate	Uniseriate and multiseriate	
Periderm	Limited	Multi-layered	
Cortex	Broad , irregular enlarged, hypertrophied individual cells, numerous intercellular spaces; loose arrangement of cortical parenchyma; frequently contain plastids with chlorophyll and other pigments, starch grains	Uniform, compact cortical cell arrangement	
Vascular bundles	Narrow, primary, immature development, limited secondary cambium activity	Cambium activity consistent	
Xylem and Phloem	Poorly developed; multiple scattered xylem bundles with variable stele patterns, no or limited secondary xylem growth; phloem frequently contain plastids with chlorophyll and other pigments, starch grains	Secondary xylem formation arranged in diarch, triarch or pentarch stele patterns in different species.	
Root hairs	Few or no, thick, short and straight, fine, delicate appearance, many fused together, usually abundant	Long, thin, slender, fibrous, wiry and formed an interwoven mat;	
STEMS	Small diameter	Large diameter	
Epidermis	Limited development	Fully developed	
Cortex	Limited development, little collenchyma, few sclerenchyma fibers; starch grains in old stem.	Fully developed with continuous cylinder of collenchyma	
Vascular bundles	Poorly developed; lower activity of cambium or not initiated; large number of starch grains in the vascular bundles at the early stages	Fully developed	
Xylem and Phloem	Roots frequently initiated in densely cytoplasmic phloem parenchyma; more xylem and phloem vessels in easy-to-root species; starch grains in the parenchymatous cells of the xylem and phloem at early stages.	Thick layers of sclerenchyma in the phloem of the stele.	
Pith	Limited development, thin cell walls; starch grains in older stems.	Fully developed, having both thin and thick cell walls	
Callus	Frequently accompanies root formation	Usually not produced	
Root-to-shoot vascular connection	The connections were often discontinuous in maple, birch and apple (*Malus domestic* Borkh – "McIntosh"); poorly developed in cauliflower. In jasmine, *Pinus*, spruce, apple (*Malus domestic* Borkh – "Jork") vascular connection were observed.		

Smith *et al.,* (1991) compared anomalous *in vitro* and *extra vitrum* root structure in various woody plants (*Ace*r *rubrum* L., *Betula nigra* L., *Forsythia x intermedia* Zab., *Malus x domestica* Borkh, *Rosa chinensis minima*). It was found that *in vitro* roots contained enlarged, irregular, frequently pigmented cortical cells with large, hypertrophied individual cells and numerous intercellular spaces and only a primary vascular system. However, *extra vitrum* roots were comprised of smaller and uniformly

arranged cell layers, exhibiting the development of a non-pigmented secondary vascular system. Furthermore, these studies (Smith *et al.,* 1991) indicated that in *ex vitro* roots the vascular cambium activity and secondary xylem formation were consistently arranged in either diarch, triarch, or pentarch stele patterns, depending on the species. By contrast, roots developed *in vitro* were found to have, an immature vascular system, usually with multiple scattered xylem bundles having varied stele patterns and no secondary growth. Secondary tissue development *in vitro* was not only temporarily delayed as compared to plants that went through *extra vitrum* rooting but. the vascular system of 10–week-old *in vitro* plants was underdeveloped. Vascular cambium activity was not initiated in *in vitro* roots until the rooted microcuttings were transplanted under *extra vitrum* conditions. Exposure to light was found to induce anomalous structure in *in vitro* roots. When light was partially excluded from the *in vitro* rooting zone, the resultant root anatomy was more uniform than in light-grown roots, but vascular system development remained inhibited as compared to *ex vitro* roots. Chlorophyll and other pigments, and large amyloplasts were observed only in roots fully exposed to light during the initiation phase.

Table 13.2 Anatomical and histological differences between the leaves of plants *in vitro* and after transplanting *ex vitro*

Plant Organ and Tissue	*In vitro*	*Ex vitro*	Species studied
LEAVES	Small, succulent, brittle and hyperhydrated	Normal shape and size	**Leaves**: *Actinidia deliciosa* *Brassica oleracea botrytis* *Brassica oleracea capitata* *Chrysanthemum morifolium* *Cucumis melo* *Cydonia oblonga* *Cynara scolymus* *Dianthus caryophyllus* *Dieffenbachia maculata* *Eucalyptus grandis* x *E. europhylla* *Fragaria* x *ananas* *Gerbera jamesonii* *Gladiolus grandiflorus* *Liquidambar styraciflua* *Maranta leuconeura* *Nephrolepis exaltata* *Olea europaea* *Pelargonium zonale* *Philodendron* Burgundy *Picea sitchensis* *Prunus amygdalus* *Prunus avium* *Prunus cerasus* *Prunus insititia* *Pyrus communis* *Pyrus malus* *Rubus idaeus.* *Salix babylonica* *Solanum laciniatum* *Spathiphyllum floribundum* *Spathiphyllum walliissii*
Epidermis	Deformed thin cell walls irregularly shaped	Normal cell walls	
Cuticle	Thin and discontinuous	Thick and continuous	
Stomata	Irregular guard cells with thin cell walls, large stoma	Normal guard cells	
Mesophyll	Irregular structure mainly spongy parenchyma	Regular structure	
Palisade	Reduced palisade tissue	Normal palisade	
Spongy Parenchyma	Highly vacuolated with large intercellular air-spaces	Normal cells with regular air-spaces	
Vascular bundles	Immature and limited vascular development	Fully developed	
Phloem	Limited secondary phloem, abundant starch grains	Normally oriented sieve tubes	
Xylem	Reduced lignification in the xylem vessels and sieve elements	Fully developed xylem vascular tissue	
Chloroplasts	Low chlorophyll, abnormal non-functional chloroplasts, limited formation of grana, abundant stroma, starch grains.	Normal chloroplasts with typical granal structure, increased chlorophyll content	

A comparison by Soukup *et al.* (2004) of the lignin content in roots of wild cherry and pedunculate oak, indicated that cell wall lignification increased in both species during the acclimatization period and reached levels similar to seedling grown in soil. In cherry roots cultured *ex vitro* phi-thickenings were observed in cortical layers just outside the endodermis, while suberised exodermis was present in oak but not in cherry, indicating a different response to acclimatization in the two species.

Adventitious roots grown *in vitro* frequently have a few or no root hairs (Preece and Sutter 1991). In Asian jasmine (*Trachelospermum asiaticum*), it was found that *in vitro* adventitious root hairs were morphologically thicker and their length was shorter by one-third to one-half of *extra vitrum* root hairs. However, there were no apparent differences in root-hair density between *in vitro* and *extra vitrum* plants. Morphologically, *in vitro* root hairs were shorter, thicker and straight compared to *extra vitrum* root hairs that were long, thin, wiry and interwoven as a mat (Apter *et al.,* 1993).

Petioles of red raspberry plants that developed *in vitro* lacked collenchyma and had greatly reduced phloem fibers in comparison with field-grown plants (Donnelly *et al.,* 1985). The vascular tissue of micropropagated birch leaves was greatly reduced in the midribs and petiole compared with leaves of greenhouse-grown trees (Smith *et al.,* 1986).

During acclimatization persistent leaves of strawberry plants became thicker due to enlargement of the palisade cells (Fabbri *et al.,* 1986), but there was no change in the number of layers of palisade cells, or in the amount of intercellular air spaces in the mesophyll. New leaves that formed during acclimatization had multiple palisade layers and, at a later stage, the youngest leaves became similar to those of field-grown plants.

Only new leaves that were fully formed after removal from culture resembled greenhouse-grown leaves (Grout and Aston, 1978; Brainerd and Fuchigami, 1981; Wetzstien and Sommer, 1982; Donnelly *et al.,* 1985). The culture atmosphere during the last stage prior to transplanting can affect the anatomy of plants as was shown for carnation by Majada *et al.,* (2001). Ventilation modified the anatomical characteristics of shoots and leaves as compared to plants grown in non-ventilated vessels: the cuticle became thicker and the supportive tissue had thicker cell walls. Anatomical variability of *in vitro* grown plants was demonstrated to a consequence of ventilation.

Hydathodes are structures related to the leaf water status that are affected by humidity and culture condition. Differences were observed between *in vitro* and *extra vitrum* leaves. In micropropagated blackberry (Donnelly *et al.,* 1987) and strawberry (Donnelly and Skelton, 1987), the hydathodes on the leaves were open, whereas those on leaves of greenhouse-grown plants were closed or had smaller apertures. Since the hydathodes appeared to be open and exhibited guttation, it was hypothesized that they might contribute to adaxial water loss.

Increasing light intensity in shoots of Kiwifruit (*Actinidia deliciosa*) plants cultured *in vitro*, resulted in an increase in the relative size of leaf mesophyll cells, and in a decrease in the number of chloroplasts and starch grains that they contained. Addition of sucrose to the substrate media had a negative effect on the size of the mesophyll cells under normal Mg concentration, but a positive effect under high Mg concentration (a 3-fold increase) (Dimassi and Bosabalidis, 1997).

In *Prunus avium* light and electron microscopy revealed that hyperhydric leaves had a lower number of chloroplasts in the palisade. The chloroplasts showed an irregularly arranged membrane system with evagination of different sizes along the plastid envelope (Frank *et al.,* 1997). The laminar midrib of normal eggplant leaves contained vascular bundles with lignified xylem elements, while in hyperhydric leaves the vascular bundles were poorly lignified. The cell density observed was 185/μm in normal and 57/μm in compact hyperhydric leaves (Picoli *et al.,* 2001).

Anatomical study of *in vitro* and *ex vitro* rooting process of *Prunus davidopersica* "Piroska" revealed that *in vitro* leaves were more loose and spongy at the end of rooting process. In the first newly developed leaf of an acclimatized *in vitro*-grown plant, the parenchyma was more developed and contained fewer rows of cells and air space as compared to leaves of field-grown plants. The *in vitro* developed roots had a broad cortex and a narrow vascular cylinder with less developed xylem elements. The vascular system became prominent in the roots at the end of the acclimatization phase (Jambor *et al.,* 2001).

5. CONCLUSION

Normal plant development *in vitro* can be manipulated by controlling the microenvironment: humidity in the head space, water and osmotic potential in the medium, and the temperature and irradiance (George 1996). A well balanced mineral nutrient composition, adequate supply of carbohydrates and levels of growth regulators in the medium, are major factors in morphogenetic

expression *in vitro*. In general, the *in vitro* conditions should mimic, as far as possible the optimal growth *extra vitrum* conditions. Acclimatization *in vitro* prior to transplanting, exposing the plants to semi-stress conditions in the third culture stage, and the use of low levels of growth retardants to alleviate excessive growth and reduce hyperhydricity has been recommended (Ziv, 1992).

REFERENCES

APTER R.C., MCWILLIAMS E.L. & DAVIS F.T. JR. 1993 *In vitro* and *ex vitro* adventitious root formation in Asian jasmine (*Trachelospermum asiaticum*) I. Comparative morphology. J. Am. Soc. Hortic. Sci. 118, 902-905.

BLANKE M.M. & BELCHER A.R. 1989 Stomata of apple leaves cultured *in vitro*. Plant Cell Tissue Organ Cult. 19, 85-89.

BORNMAN C.H. & VOGELMANN T.C. 1984 Effect of rigidity of gel medium on benzyladenine-induced adventitious bud formation and vitrification in *in vitro Picea abies*. Physiol. Plant 61, 505-512.

BRAINERD K.E. & FUCHIGAMI L.H. 1981 Acclimatization of aseptically cultured apple plants to low relative humidity. J. Am. Soc. Hortic. Sci. 106, 515-518.

CAPELLADES M., FONTARNAU R., CARULLA C. & DEBERGH P. 1990 Environment influences anatomy of stomata and epidermal cells in tissue-cultured *Rosa moltiflora*. J Am. Soc. Hortic. Sci. 115, 141–145.

CAPELLADES M., LEMEUR R. & DEBERGH P. 1991 Effects of sucrose on starch accumulation and rate of photosynthesis in *Rosa* cultured *in vitro*. Plant Cell Tissue and Organ Cult.25, 21-26.

CONNER A.J. & THOMAS M.B. 1981 Re-establishing plantlets from tissue culture: A review. Proc. Intl. Plant Prop. Soc. 31, 342-357.

CONNER L.N. & CONNER A.J. 1984 Comparative water loss from leaves of *Solanum laciniatum* plants cultured *in vitro* and *in vivo*. Plant Sci. Lett. 36, 241-246.

DAMI I. & HUGHES H.G. 1997 Effects of PEG-induced water stress on *in vitro* hardening of 'Valiant' grape. Plant Cell Tissue Organ Cult. 47, 97-101.

DE KLERK G.J., KEPPEL M., TER BRUGGE J. & MEEKES J. 1995 Timing of the phases in adventitious root formation in apple microcuttings. J. Exp. Bot. 46, 965-972.

DE KLERK G.J., VAN DER KRIEKEN W. & DE JONG J. 1999 The formation of adventitious root: New concepts, new possibilities. In Vitro Cell Dev. Biol. Plant 35, 189-199.

DIMASSI-THERIOU K. & BOSABALIDIS A.M. 1997 Effects of light, magnesium and sucrose on leaf anatomy, photosynthesis, starch and total sugar accumulation, in kiwifruit cultured *in vitro*. Plant Cell Tissue Organ Cult. 47, 127-134.

DONNELLY D.J. & SKELTON F.E. 1987 Hydathode structure of micropropagated plantlets and greenhouse grown 'Totem' strawberry plants. J. Am. Soc. Hortic. Sci. 112, 755-759.

DONNELLY D.J. & VIDAVER W.E. 1984 Leaf anatomy of red raspberry transferred from *in vitro* culture to soil. J. Am. Soc. Hortic. Sci. 109, 172-176.

DONNELLY D.J., SKELTON F.E. & NELLES, J.E. 1987 Hydathode anatomy and adaxial water loss in micropropagated 'Silvan' blackberry. J. Am. Soc. Hortic. Sci. 112, 566-569.

DONNELLY D.J., VIDAVER W.E. & LEE K.Y. 1985 The anatomy of tissue cultured red raspberry prior to and after transfer to soil. Plant Cell Tissue Organ Cult. 4, 43-50.

ESAU K. 1977 *Anatomy of Seed Plants* (2nd ed.) Wiley, New York.

FABBRI A., SUTTER E. & DUNSTON S.K. 1986 Anatomical changes in persistent leaves of tissue cultured strawberry plants after removal from culture. Sci. Hortic. 28, 331-337.

FRANCK, T., CREVECOEUR, M., WUEST, J., GREPPIN, H. AND GASPAR, T. 1997 Cytological comparison of leaves and stems of *Prunus avium* L. shoots cultured on a solid medium with agar or gelrite. Biotechnic & Histochemistry 43,32-43.

FUCHIGAMI L.H., CHENG T.Y. & SOELDNER A. 1981 Abaxial transpiration and water loss in aseptically cultured plums. J. Am. Soc. Hortic. Sci. 106, 519-522.

GEORGE E.F. 1996 *Plant Propagation by Tissue Culture.* Part 2. *In Practice.* Exegetics Ltd. Edington, Wesbury, UK.

GRONROOS R. & VON ARNOLD S. 1985 Initiation and development of wound tissue and roots on hypocotyl cuttings of *Pinus sylvestris in vitro*. Physiol. Plant. 64, 393-401.

GROUT B.W.W. 1975 Wax development on leaf surface of *Brassica*o *oleracea botrytis* cv. Currawon regenerated from meristem culture. Plant Sci. Lett. 5, 401-405.

GROUT B.W.W. & ASTON H. 1977 Transplanting of cauliflower plants regenerated from meristem culture. I. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. Hortic. Res. 17, 1-7.

GROUT B.W.W. & ASTON H. 1978 Modified leaf anatomy of cauliflower plantlets regenerated from meristem culture. Ann. Bot. 42, 993-995.

HACKETT W.P. 1985 Juvenility, maturation, and rejuvenation in woody plants. Hortic. Rev. 7, 109-155.

HARBAGE J.F., STIMART D.P. & EVERT R.F. 1993 Anatomy of adventitious root formation in microcuttings of *Malus domestica* Borkh. "Gala". J. Am. Soc. Hortic. Sci. 118, 680-688.

HICKS G.S. 1987 Adventitious rooting of apple microcuttings *in vitro*: An anatomical study. Can. J. Bot. 65, 1913-1920.

ILIEV I., KITIN P. & FUNADA R. 2001 Morphological and anatomical study on *in vitro* root formation of silver birch (*Betula pendula* Roth.). Prop. Orn. Plants 1, 10-19.

ILIEV I., RUBOS A., SCALTSOYIANNES A., NELLAS H. & KITIN P. 2003 Anatomical study of *in vitro* obtained fasciated shoots from *Betula pendula* Roth. Acta Hortic. 616, 481-484

JAMBOR B.E., KISSIMON J., FABIAN M., MESZAROS A., SINKO Z., GAZDAG G. & NAGY T. 2001 *In vitro* rooting and anatomical study of leaves and roots of *in vitro* and *ex vitro* plants of *Prunus* x *davidopersica* "Piroska". Int. J. Hort. Sci. 7, 42-46.

JASIK J. & DE KLERK G.J. 1997 Anatomical and ultrastructural examination of adventitious root formation in stem slices of apple. Biol. Plant. 39, 79-90.

KEVERS C. & GASPAR TH. 1986 Vitrification of carnation *in vitro*: Changes in water content, extracellular space, air volume and ion levels. Physiol. Veg. 24, 647-653.

KEVERS C., FRANCK T., STRASSER R.J., DOMMES J. & GASPAR TH. 2004 Hyperhydricity of micropropagated shoots: A typically stress-induced change of physiological state. Plant Cell, Tissue Organ Cult. 77, 181-191.

KOSHUCHOWA S., BOTTCHER I., ZOGLAUER K. & GÖRING H. 1988 Avoidance of vitrification of *in vitro*-cultured

plants. *in* Proceedings 6th Congress of FESPP (Abstract p. 1418), Split, Yugoslavia

LEE N., WETZSTEIN H.Y. & SOMMER H.E. 1985 Effect of quantum flux destiny on photosynthesis and chloroplast ultrastructure in tissue-cultured plantlets and seedlings of *Liquidambar styraciflua* L. towards improved acclimatization and field survival. Plant Physiol. 78, 637-641.

LEE N., WETZSTEIN H.Y. & SOMMER H.E. 1988 Quantum flux density effects on the anatomy and surface morphology of *in vitro* and *in vivo* developed sweet gum leaves. J. Am. Soc. Hortic. Sci. 113, 167-171.

LESHEM B. 1983 Growth of carnation meristems *in vitro*: Anatomical structure of abnormal plantlets and the effect of agar concentration in the medium on their formation. Ann. Bot. 52, 413-415.

LESHEM B., WERKER E. & SHALEV D.P. 1988 The effect of cytokinins on vitrification in melon and carnation. Ann. Bot. 62, 271-276.

LOURO R.P., DOS SANTOS A.V. AND MACHADO R.D. (1999) Ultrastructure of *Eucalyptus grandis* x *Eucalyptus urophylla*. I. Shoots cultivated *in vitro* in multiplication and elongation-rooting media. Int. J. Plant Sci. 160, 217-227.

LOVELL P. L. & WHITE J. 1986 Anatomical changes during adventitious root formation. pp. 111-140 *in* Jackson M.B. (ed.) *New Root Formation in Plants and Cuttings*. Martinus Nijhoff Publ. ISBN 90 247 3260 3.

MAJADA J.P., TADEO F., FAL M.A. & SANCHEZ-TAMES R. 2001 Impact of culture vessel ventilation on the anatomy and morphology of micropropagated carnation. Plant Cell Tissue Organ Cult. 63, 207-214.

MARIN J.A., GELLA R. & HERRERO M. 1988 Stomatal structure and functioning as a response to environmental changes in acclimatized micropropagated *Prunus cerasus* L. Ann. Bot. 62, 663-670.

McCLELLAND M.T., SMITH M.A.L. & CAROTHERS Z.B. 1990 The effects of *in vitro* and *ex vitro* root initiation on subsequent microcutting root quality in three woody plants. Plant Cell Tissue Organ Cult. 23, 115-123.

OLMOS E. & HELLIN E. 1998 Ultrastructural differences of hyperhydric and normal leaves from regenerated carnation plants. Sci. Hort. 75, 91-101.

PALEVITZ B.A. 1981 The structure and development of stomatal cells. pp.1-230 *in* Jarvis P.G. and Mansfield T.A. (eds.) *Stomatal Physiology*, Cambridge University Press, Cambridge.

PAQUES M. & BOXUS P. 1987 Vitrification: A phenomenon related to tissue water content. Acta Hortic. 212, 245-252.

PATEL K.R., RUMARY C. & THORPE T.A. 1986 Plantlet formation in black and white spruce. III. Histological analysis of *in vitro* root formation and the root-shoot union. N.Z.L. For. Sci. 16, 289-296.

PICOLI E.A.T., OTONI W.C., FIGUEIRA M.L., CAROLINO S.M.B., ALMEIDA R.S., SILVA E.A.M., CARVALHO C.R. & FONTES E.P.B. 2001 Hyperhydricity in *in vitro* eggplant regenerated plants: Structural characteristics and involvement of BiP (Binding Protein). Plant Sci. 160, 857-868.

PIERIK R.L.M. 1987 *In Vitro Culture of Higher Plants*. Martinus Nijhoff, Dordrecht, The Netherlands.

PINKER I. 2000 Characterization of stem quality in relation to rooting process in *Prunus* and *Amelanchier* cultures. Acta Hortic. 530, 387-395.

PREECE J.E. & SUTTER E. 1991 Acclimatization of micropropagated plants to the greenhouse and field. pp. 71-79 *in* Debergh P.C. and Zimmerman R.H. (eds.) *Micropropagation: Technology and Application*. Kluwer Academic Publishers, Dordrecht.

RANJIT M., KESTER D.E. & POLITO V.S. 1988 Micropropagation of cherry rootstocks: III. Correlations between anatomical and physiological parameters and root initiation. J. Am. Soc. Hortic. Sci. 113, 155-15

REUTHER G. 1988 Comparative anatomical and physiological studies with ornamental plants under *in vitro* and greenhouse conditions. Acta Hortic. 226, 91-98.

ROBERTS A.V., WALKER S., HORAN I., SMITH E.F. & MOTTLEY J. 1992 The effect of growth retardants, humidity and lighting at Stage III on Stage IV of micropropagation in chrysanthemum and rose. Acta Hortic. 319, 153-158.

ROGERS R.B. & SMITH M.A.L. 1992 Consequences of *in vitro* and *ex vitro* root initiation for miniature rose production. J. Hort. Sci. 67, 535-540.

SAEBO A., KREKLING T. & APPELGREN M. 1995 Light quality affects photosynthesis and leaf anatomy of birch plantlets *in vitro*. Plant Cell Tissue Organ Cult. 41, 177-185.

SAMARTIN A., VIEITEZ A.M. & VIEITEZ E. 1986 Rooting of tissue cultured camellias. J. Hortic. Sci. 61, 113-120.

SMITH M.A.L., EICHORST S.M. & ROGERS R.B. 1992 Rhizogenesis pretreatments and effects on microcuttings during transition. Acta Hortic. 319, 77-82.

SMITH M.A.L., MCCLELL& M.T. & TIMMERMANN R. 1991 Anomalous root structure on woody plants *in vitro*. J. Environ. Hortic. 9, 61-64.

SMITH M.A.L., PALTA J.P. & MCCOWN B.H. 1986 Comparative anatomy and physiology of microcultured, seedlings and greenhouse-grown Asian white birch. J. Am. Soc. Hortic. Sci. 111, 437-442.

SOLOMON M., GEDALOVICH E., MAYER A.M. & POLJAKOFF-MAYBER A. 1986 Changes induced by salinity to the anatomy and morphology of excised pea roots in culture. Ann. Bot. 57, 811-818.

SOUKUP A., MALA J., HRUBCOVA M., KALAL J., VOTRUBOVA O. & CVIKROVA M. 2004 Differences in anatomical structure and lignin content of roots of pedunculate oak and wild cherry-tree plantlets during acclimation. Biol. Plant. 48, 481-489.

SUTTER E.G. 1985 Morphological, physical, and chemical characteristic of epicuticular wax on ornamental plants regenerated *in vitro*. Ann. Bot. 55, 321-329.

SUTTER E.G. 1988 Stomatal and cuticular water loss from apple, cherry, and sweetgum plants after removal from *in vitro* culture. J. Am. Soc. Hortic. Sci. 113, 234-238.

SUTTER E.G. & LANGHANS R.W. 1979 Epicuticular wax formation on carnation plantlets regenerated from shoot tip culture. J. Am. Soc. Hortic. Sci. 104, 493-496.

SUTTER E.G. & LANGHANS R.W. 1982 Formation of epicuticular wax and its effect on water lost in cabbage plants regenerated from shoot-tip cultures. Can J. Bot. 60, 2896-2902.

VIEITEZ A.M., BALLESTER A., SAN-JOSE M.C. & VIEITEZ E. (1985) Anatomical and chemical studies of vitrified shoots of chestnut regenerated *in vitro*. Physiol. Plant. 65, 177-184.

WARDLE K., DOBBS E.B. & SHORT K.C. 1983 *In vitro* acclimatization of aseptically cultured plantlets to humidity. J. Am. Soc. Hortic. Sci. 108, 386-38.

WARDLE K., QUINLAN A. & SIMPKINS I. 1979 Abscisic acid and the regulation of water loss in plantlets of *Brassica oleracea* L var *botrytis* regenerated through apical meristem culture. Ann. Bot. 43, 745-752.

WELANDER M. & PAWLICKI N. 1993 A model system for studying root regeneration in woody species. Acta Hortic. 336, 225-230.

WERKER E. & LESHEM B. 1987 Structural changes during vitrification of carnation plantlets. Ann. Bot. 59, 377-385.

WETZSTEIN H.Y. & SOMMER H.E. 1982 Leaf anatomy of tissue cultured *Liquidambar styraciflua* (Hamamelidaceae) during acclimatization. Amer. J. Bot. 69, 1579-1586.

WETZSTEIN H.Y. & SOMMER H.E. 1983 Scanning electron microscopy of *in vitro* cultured *Liquidambar styraciflua* plantlets during acclimatization. J. Am. Soc. Hortic. Sci. 108, 475-480.

ZIMMERMAN R.H. & FORDHAM I. 1985 Simplified method for rooting apple cultivars *in vitro*. J. Am. Soc. Hortic. Sci. 110, 34-38.

ZIV M. 1986 *In vitro* hardening and acclimatization of tissue culture plants. pp. 187-196 in Withers L.A. and Alderson P.G. (eds.). *Plant Tissue Culture and its Agricultural Applications*. Butterworths, London.

ZIV M. 1991 Vitrification: Morphological and physiological disorders of *in vitro* plants. pp. 45-69 *in* Debergh P.C.and Zimmerman R.H. (eds.) *Micropropagation. Technology and Application.* Kluwer Academic Publishers, The Netherlands.

ZIV M. 1992 The use of growth retardants for regulation and acclimatization of *in vitro* plants. pp. 809-817 *in* Karssen M., van Loon L.C. and Vreugdenhil D. (eds.), *Progress in Plant Growth Regulation*. Kluwer Academic Publishers, Dordrecht.

ZIV M. 1995 *In vitro* acclimatization. pp. 493-516 in Aitken-Christie J., Kozai T. and Smith M.A.L. (eds.) *Automation and Environmental Control in Plant Tissue Culture*. Kluwer Academic Publishers, Dordrecht.

ZIV M. 1999 Developmental and structural patterns of *in vitro* plants. pp. 235-253 *in* Soh W.Y. and Bhojwani S.S. (eds.) *Morphogenesis in Plant Tissue Cultures*. Kluwer Academic Publishers, Dordrecht.

ZIV M. & ARIEL T. 1992 On the relation between vitrification and stomatal cell wall deformity in carnation leafs *in vitro*. Acta Hortic. 314, 121-129.

ZIV M. & ARIEL T. 1994 Vitrification in relation to stomatal deformation and malfunction in carnation leaves *in vitro*. pp. 143-154 *in* Lumsden P.J., Nicholas J.R. and Davies W.J.(eds.), *Physiology, Growth and Development of Plants in Culture*. Kluwer Academic Publishers, Dordrecht.

ZIV M., MEIR G. & HALEVY A.H. 1981 Hardening carnation plantlets regenerated from shoot tips cultured *in vitro*. Environ. Exp. Bot. 21, 423.

ZIV M., MEIR G. & HALEVY A.H. 1983 Factors influencing the production of hardened glaucous carnation plants *in vitro*. Plant Cell Tissue Organ Cult. 2, 55-60.

ZIV M., SCHWARTS A. & FLEMINGER D. 1987 Malfunctioning stomata in vitreous leaves of carnation (*Dianthus caryophllus*) plants propagated *in vitro*; implications for hardening. Plant Sci. 52, 127-134.

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